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A REVIEW OF EMERGING ANALYTICAL TECHNIQUES FOR STANDARDIZATION OF HERBAL MEDICINAL PRODUCTS

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Abstract: Herbal Medicine Products (HMP) are now in limelight across the globe by virtue of their particular theory and long history of practice. But inadequate and uncontrolled quality control procedures are major hurdles in their official recognition and prevalence as an established medical system. During hey days of herbal drugs, herbal practitioners carefully formulate and dispense the drug according to need of individual patient. But today, commercialization scenario has changed much. HMP are being manufactured on commercial scale where manufacturer have to tackle different problems. For instance, availability of good quality of raw material and its authentication, provision of quality standards, instruments for standardization of either single or poly herbal medicine, assurance of quality from vary step of propagation of herbs to packaging of final dosage form. The scope of this review encompasses analytical techniques especially spectrophotometric and chromatographic fingerprint methods of analysis for standardization of HMP. Furthermore, evaluation techniques of bioactive markers from herbal compounds through bio-chromatographic and conventional chromatographic procedures. Such as, fingerprint and multi-constituents quantification in particular and coupling of chemical and biological approaches such as biofingerprint and metabolic fingerprint in general are discussed here. In summary, the analysis and standardization of HMP is in progress to better address the inherent holistic nature of HMP.

Keywords: Herbal Medicine Products (HMP), quality control, bioactive markers, chromatography fingerprint

Herbal medicines are widely accepted way of treatment in both developed and developing countries, WHO revealed that 80% of world population still uses herbs and other traditional medicine for their primary ailments (1). A 2012 survey revealed that 32.2% of adult population consumes Herbal Medicine Products (HMP) in the United States of America (2). Moreover, this trend is not confined to North America, even in Scandinavian countries, for instance, in Norway 39.7% of 600 surveyed pregnant women depend upon herbal products, most commonly ginger, iron-rich herbs, Echinacea and cranberry (3). Although a very diverse range of herbal medicines are in use across the continents, but unfortunately the traditional herbal products have not been officially recognized. This indifference towards herbal products might be due to procrastination in this field or because of insufficient research. The quality, safety and efficacy data to legitimize the herbal drug according to western medicine regulatory authorities is inadequate and not up to mark in comparison with prevailing allopathic medicinal system. The main enigma of herbal medicine is their assorted chemical composition, which is due to multiple factors, these factor varies from botanical species, chemo types, parts in use (root, rhizomes, twigs, seeds leaf etc.). In addition, process of collection, drying, curing, atmospheric moisture, day latitude, altitude, variability in harvesting and geographical conditions are also responsible for variation in contents of herbal drugs (4). In a nutshell a herbal medicine is diverse in its chemical composition from batch to batch, which eventually leads to significant pharmacological activity variation, this activity divergence may lead to pharmacodynamics variability or pharmacokinetics difference within the same batch or in different batches of same finished herbal product (5). In order to overcome these possible pitfalls in safety, efficacy, authentication and for establish-
ment of rationality between the herbal component and conventional usage of HMP a multidisciplinary approach is pivotal (6). Getting beneficial fact from samples with heterogeneous chemical constituents has long been a herculean task. Herbal formulations are comprised of numerous chemical compounds of variety of chemical structures, among them merely few, if not single, have been recognized by scientific screening, which are responsible for either therapeutic and/or toxic potential (7). Standardization of herbal products through spectrophotometric and chromatographic fingerprint analysis is quite successful to resolve this quality control riddle.

Standardization of HMP

Standardization interpreted by American Herbal Product Association as “Standardization refers to the body of information and controls necessary to produce material of reasonable consistency. This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing”. Furthermore, standardization eliminates or reduces batch to batch variation; ensure safety efficacy, quality and acceptability of HMP. A herbal product cannot be recognized scientifically effectual if drug test has not been established and characterized to ascertain reproducibility in manufacturing process (8).

The active pharmaceutical ingredients (API) of herbal medicinal products are generally defined to be the whole herbal preparation, e.g., the extract in its eternity (9). Individual or groups of constituents have only been identified in selected cases to be onus for therapeutic activity. As the whole herbal product, e.g., extract, is regarded as the active pharmaceutical ingredient. Several extracts types depending on toxicological, pharmacological, pharmaceutical analytical and clinical findings, could be discriminated into 3 categories (A, B1 and B2) (9). Extracts of Type A: this type contains either single or groups of constituents which are solely acknowledged for recognized therapeutic activity. Standardization of a defined content is acceptable. In Chinese Herbal Pharmacopeia quality of 529 herbs out of recorded 1203 raw materials of poly herbal formulations is based on the use of single evaluation marker (10).

Extracts Type B1 contains well defined chemical constituents either single or groups which show relevant pharmacological properties (active markers). Active markers are likely to be responsible for clinical efficacy, however, evidences that they are the only capable constituents in their respective HMP for clinical efficacy need evidences hitherto. The available data of efficacy, quality and safety of an extract should be taken into consideration (9).

Extract Type B2 containing neither single nor groups of constituents responsible for efficacy, or relevant pharmacological activity. To standardize these types of compounds chemically defined constituents (markers) without known therapeutic activity may be useful as a standard. These markers also serve to ensure good manufacturing practice or as bases for content assay of drug products (9). However, for most poly herbal medicines, the chemical or therapeutic components have not been fully elucidated or easily monitored.

Fingerprint analyses fill this void to some extent, since it emphasizes more of the characteristic of poly herbal drug formulations. Fingerprint is a characteristic profile or pattern which chemically interpret the sample composition and in which, usually, maximum information is depicted. By and large, fingerprints could be obtained through several techniques, both chromatographic and spectroscopic (11). Fingerprint method is widely accepted analytical standards by different official or non-official organizations like World Health Organization (12), British Herbal Medicine Association (13), German Commission E (14), and European Medicine Agency (15). Furthermore, compulsory fingerprint analysis is purposed requirement for Chinese herbal products from Chinese State Food and Drug Administration since 2004 (16).

Fingerprint analysis addresses two main issues; 1. How to attain authentic and effective information? 2. How to determine similarity with chromatographic methods?

Generally, chromatographic fingerprint process is executed by determination of common and stable compound by screening out large number of samples by utilizing different chromatographic and separation techniques.

Hyphenated detection methods

Detection of the target compounds is subsequent step after sample separation. For detection, hyphenation of different techniques include ultraviolet (UV) or diode-array detection (DAD), evaporative light scattering detection (ELSD), charged aerosol detection (CAD), mass spectroscopy and enzyme-linked immunosorbent assay (MS & ELISA). A combination of multiple techniques could provide relative analytical data for instance diode-array detection – evaporative light scattering
A review of emerging analytical techniques for standardization...

detection (DAD–ELSD) and diode-array detection–mass spectrometry (DAD-MS).

**Liquid chromatography – diode-array detection/mass spectrometry (LC-DAD/MS)**

It is a recommended technique for determination of unspecified compounds by comparison with standards. Ding and colleagues have determined the alkaloids in *Corydalis yanhusuo* using LC-MS/MS and LC-DAD (18). In addition, Lee et al. used LC-MS for quick identification of aristolochic acid content in plant medicines (19). LC-NMR has distinct superiority to unambiguously identify the structures of compounds. However, this method has some downsides, such as relatively low sensitivity and high cost.

**Ultraviolet (UV) or diode-array detection (DAD)**

Since its inception, UV is most pervasive and mainstream analytical technology. It is ubiquitous in laboratories, inexpensive, and easy to use. Nevertheless, it has its downsides; poor absorbance decrease sensitivity of UV due to intervention of short wavelength compounds. In addition, the range of mobile phase is narrow and option for selection of modifiers is also restricted. UV integrated successfully with liquid chromatography and capillary electrophoresis for herbal products. DAD is now more prevalent than UV due to its ability to measure wide range of wavelengths and capability to deliver instant on-the-fly spectrum with the edge of enhanced sensitivity and peak precision (20).

Contemporarily, UV/DAD effectively integrated with mass spectrometry. UV/DAD–MS techniques for quick qualitative and quantitative analysis of HMP. Fan et al. determined ten major active components in *Carthamus tinctorius* L. to carry out a qualitative and quantitative evaluation based on HPLC-DAD. The relative standard deviation peak area for each of the ten bioactive compounds was calculated, respectively, to validate the precision of the method (21).

**Evaporative light scattering detection (ELSD)**

ELSD is pervasive, nonspecific analytical technique that provides a steady starting line even with gradient elution (22). In addition, for enhanced discrimination of analyte constituents, volatile mobile phase modifiers, for example, formic acid and acetic acid are successfully used (23). An HPLC–ELSD method has been developed for simultaneous monitoring of 12 ginseng saponins in different parts of *P. quinquefolia* (24). 14 saponins in red *P. ginseng*, (25) and 19 saponins in black ginseng (26).

**Charged aerosol detection (CAD)**

Nevertheless, ELSD is an inappropriate technique for detection of non UV, weakly UV absorbing, and UV range absorbing samples without reference standard. In such cases charged aerosol detection remains method of choice. Mass is working principle for either charged aerosol detection or ELSD. This technique has a distinct, that neither physiochemical nor spectral properties of analyte could affect final result. With an identification method that produce universal response factors, there is potential for a single universal standard for calibration against which all other compounds or impurities can be qualified (27). CAD has an edge of increased sensitivity over ELSD system, broader dynamic selection, convenient operation and uniformity of response elements. Bai et al. successfully maneuvered a HPLC-CAD for the simultaneous determination of seven different saponins in *Radix et Rhizoma Notoginseng* (28).

**Recent advancements**

Although manipulating several active ingredients as evaluation markers is an effective technique, but multicomponent composition of herbal medicine render their separation and screening substantially difficult, moreover many plants share the same compounds which further complex their standardization. This method might be inadequate to confirm the identity of specific plants. To curb this limitation more feasible techniques are needed. Following advances address these issue to certain extent.

**Multiple-patterns of chromatographic fingerprints**

However, chromatographic fingerprint acclimed as an appropriate and suitable analytical method for quality control of HMP but usually a single chromatogram is assessed to carry out this analysis. By considering complex and multi components composition of herbal product, a single chromatogram could not characterize all chemical patterns or characteristics of sample. It is practically out of question to outline an analytical method to represent all chemical features of constituents in a single chromatogram (29, 30). To solve this problem a combination of analytical methods with multiple separation techniques and analytical conditions are employed. This multi-disciplinary approach is also recognized by FDA and Chinese State Food and Drug Administration (13, 30). It became inevitable to devise a method which could integrate multiple chromatographic fingerprint to demonstrate the complex multi constituent composition of herbal
formulations for determination of quality. Fan et al. carried out investigation of Danshe Dropping Pill and Panax notoginseng by multiple chromatographic fingerprint for quality control. They opted for two extraction methods to create multiple HPLC chromatograms, then exploit retention time, UV absorbance and MS spectra for qualitative and quantitative analysis and finally a data level information fusion method is manipulated to obtain integrated chemical features of binary fingerprints. The resultant multiple fingerprints ensured lot to lot consistency and avoidance of fraudulent material by using similarity measures and by chemometric approach (29). Unequivocally, multiple chromatographic fingerprint will give detailed and precise information about plant material.

**HPLC fingerprint integrated with multiple component quantified assessment**

In contrast with synthetic drugs, it is established fact that therapeutic potential of HMP is connected with synergism of their heterogeneous compositions and different target sites on that they act upon. In chromatographic fingerprint analysis of such complex compounds an analogy is drawn between similarity and differences of single markers of various samples which demonstrate overall view of subjected herbal drug. However, one drawback of this technique is that it can only demonstrate result by showing similarity calculated on relative magnitude by using already known marker compound as a reference standard. Little variability among alike chromatograms may not be differentiated. As a consequence, multiple component analysis should be taken into account for plausible illustration of HMP. Yang et al. first time reported chromatographic fingerprint analysis and concomitant determination of eleven active compound in traditional Chinese medicine Shuang-huang-lian (SHL) oral liquid formulation by HPLC-DAD. This novel analytical method resolved the problem created by comparative analysis of single marker in sample. Moreover it provided greater qualitative information than any other singular evaluation and proved to be a simple, sensitive, accurate and reliable quality control procedure for SHL oral liquid sample (31).

**Exploitation of biochromatographic fingerprints for bioactive markers exploration**

Prevalent techniques for standardization of herbal medicine are either compound based or pattern based. Compound focused technique aims particular component having defined molecular structure and pharmacological activity. On the other hand, pattern oriented technique focuses all detectable components (either they are pharmacologically active or not). Although pattern oriented methods which utilize chemical markers serve the purpose of analysis but ideal chemical marker should be a characteristic component of known pharmacological activity to ascertain quality. Conventional way of biological screening in which isolation of chemical compound is done first then screening its activity in vitro or in vivo is carried out through animal models, organ and tissue models, cellular models or receptor and enzyme models. These techniques proved time consuming, arduous and inadequate for recognition of synergetic activities of HMP. By taking this into consideration, screening of bioactive components become inevitable for authentication of the therapeutic bases, exploration of leading compounds and for provision of appropriate chemical markers for standardization. To solve this conundrum advance techniques like bio fingerprinting chromatogram analysis are preferred, in which comparative assessment of fingerprint chromatograms of the extract of HMP prior and afterward the interaction with biological systems is carried out. In the first step, immobilization of certain biomolecule as solid phase is done to reflect the affinity interaction between the analytes and non-column targets. In the second step, to interact herbal drug with a some target macro biomolecules (DNA, protein, cell, enzyme etc) and the third step is to study the metabolism of HMP in vitro or in vivo and then analyze the metabolic fingerprint (32-35).

Isolation of each constituent of HPMs is so onerous that the aforementioned techniques are quite plausible. In addition, along with defined active components some new compounds are likely to be detected, this phenomenon also demonstrate that how HMP exert their synergistic or antagonistic potential (34). For instance, a novel approach of integrating HPLC with microanalysis sampling integrated with DNA attachment for evaluation HMP extract.

In analysis of traditional Chinese medicine seven compounds were attached to calf thymus DNA from the Coptis chinesis Franch (Coptis). But only three from Phellodendron amurense Rupr. (Phellodendron) and none from Sophora flavescens Ait to bind ct-DNA, respectively. Three of them were identified as berberine, palmatine and jatrorrhizine and their association constant (K) to ct-DNA were determined by microdialysis/HPL (34). Nevertheless, limitation of these methods should also be taken into account during carrying out of these analyses. First, each model is not appro-
appropriate for all HMP, for instance, no potential component is detected in DNA model analysis of *Sophora flavescens* (34). Ferulic acid, one of the main active compounds of DBD, could not appear in several screening methods. The parallelism of different screening methods is not perfect, so it easily creates mess. For example, the potential constituents of different Chinese medicine are different in different screening strategies (35-37). This is possible due to multi target and multi compounds characteristic of HMP. Combining with the activity verification *in vivo* or *in vitro* is done in order to find those which can precisely predict the active components. In a nutshell, bio fingerprint analyses is effective and swift approach for profiling the bioactive markers of herbal products they also demonstrate potential future for standardization of herbal products.

**Quantitative analysis of multi components by single marker (QASM)**

Provision of certified standards of evaluation markers, is not an easy task because of shortcomings in their separation, stability, maintenance and supply which render their use in assessable. To counter these shortcoming Wang et al. designed a novel method named quantitative analysis of multi components by single marker (QASM), which manipulates a single marker to concurrent detection of multiple inaccessible components because active components in plants having inherent functional and proportional relationships. The relative retention values between the target compound and single marker were used for qualitative analysis, while their relative correction factor applied to quantitative analysis (38).

**Thin layer chromatography – bio – autographic assay (TLC-BAA)**

It is a well-developed method combining TLC isolation with biologic activity screening. It can swiftly detect and distinguish bioactive constituents in a complex herbal formulation and has an edge of convenience, simplification and no need of special equipment. From very first day of introduction of anti-bacterial drug screening using paper chromatography bio-autography, it is recognized and widely acclaimed as a rapid activity-screening tool (39). Furthermore, it got acceptance for the activity screening of pure chemical entities (40) and the activity guided purification of bioactive natural compounds, for instance, antibacterial compounds (41–43), antifungal (44, 45), cholinesterase inhibitors (46), and free radical scavenging and antioxidant active ingredients (47). TLC-BBA is a swift and simple mean to compare characteristic profile and fingerprint activity of HMP. Nevertheless, due to its drawbacks, such as constraints in the polarity of the tested compounds and its sensitivity to bacteria on a TLC plate, endeavors are needed to establish TLC-BBA for broader application in quality control of HMP.

**CONCLUSION**

This review briefly describes the progress and existing standing of standardization of herbal products, especially quality control methods and technologies that have been currently introduced. These developments stipulated that standardization and analysis has already been entered to a new phase. This is not trivial to apply latest instruments either solely or in combination for quality control of centuries old medicinal products. But so far discussed in this review and whatever has been done in this regard is just embarkment on long expedition. Use of chromatographic fingerprint of herbal medicine standardization only applied to compare the relative similarities and/or differences and monitoring their stability. The complicated nexus between efficacy of HMP and chromatographic fingerprints is still a grey area. Efficacy of HMP is attributed to mixture of compounds present in the herbs, which emphasize that rational evaluation of their relation could not be neglected. HMP pose a challenge for researchers by variation in activity of single herb and complexity of poly herbal formulations. Moreover, utilization of just chemical profiling is not enough for determination of efficacy of HMP. This is area where integration of interdisciplinary approaches like biochemistry, molecular biology or cell biology might be successful. Chemical fingerprints could be linked to these biological assays to provide a prudent solution for efficacy and quality issues of HMP, but hitherto work on this aspect is too little too short to meet the necessary criteria. Thus to establish a methodological correlation between chromatographic fingerprints and efficacy of HMP is a need of hour.

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Malaria is the world’s greatest life bullying parasitic infection that impacts on the productivity of community due to morbidity and mortality. The cause of this disease is protozoan parasites of the genus *Plasmodium* that infect human hosts, and is a critical worldwide health matter with about 200 million cases and 1 million deceases in 2010 (1). The complicated lifespan of malaria parasite consists of two hosts and five tissues of host whilst having at least ten different morphological moves (2). Clinical symptoms of malaria are due to repetition of parasites, subsequent destruction of erythrocytes and most of medicines target asexual (human-host) stages of the lifespan. Clinically, manifestations of malaria are chills, anemia, fever and prostration. Severity of this disease may lead to cerebral malaria, delirium, metabolic acidosis, coma and death (3). Even after clearance of parasite from bloodstream relapse can occur because of the presence of some strains of malaria, particularly *Plasmodium vivax*, can be in a latent liver hypnozoite system (4). Major cause of severe malaria is *P. falciparum* which is a reason of majority of deaths among five relevant species of human parasites (5). Therefore, drug development efforts are concentrating on *P. falciparum*, generally at the expense of other species.

New medications with exclusive structures and appliances of action are required to overcome the drug resistance issues by continuous evolution of the parasite (6). Therefore, disease governor and treatment has been thorny by the advent of resistance to broadly used antimalarial drugs reinforcing the vital requirement for new treatments. To overcome the problem of drug confrontation, newer lead compounds and drug targets are required. Fortuitously, a large amount of reserves have been focused on the way to antimalarial lead compounds finding in the...
past limited decades and fungi are considered as an important source of antimalarial compounds.

**Antimalarial metabolites from fungi**

The metabolites produced by fungi represent a use of biosynthetic pathways, a wide range of molecular diversity, and a particular power to modulate biological processes (7). They signify one-fourth of all recognized bioactive natural products. Therefore, fungi exhibit a source for the discovery of structurally novel and diverse natural products possessing biomedical prospective (anticancer, antileishmanial, antibacterial and antimalarial). Many scientists have been investigated fungi for antimalarial metabolites production. In current review we covered details of isolation of fungal metabolites and their biological activities. Following are few of important classes of reported metabolites.

**Macrolides**

Macrolides are a collection of drugs containing a great macrocyclic lactone ring with one or more deoxy sugars. These are polyketide type of secondary metabolites. Tanaka and his coworkers studied antimalarial effects of fungal metabolite, radicicol (1), a macro cyclic lactone antibiotic (8). In another study, Isaka and his colleagues reported resorcylic macrolides aigialomycins A-E (2-6) purified from *Aigialus parvus*. Compound 5, aigialomycin D, was

Figure 1a. Fungal macrolides with antimalarial activity (1-6)

Figure 1b. Fungal macrolides with antimalarial activity (7-14)
found moderately active agent against *P. falciparum* which showed IC_{50} value of 6.6 µM/L (9).

Rukachaisirikul and his coworkers in 2004 purified three new macrolides (7-9) along with six recognized compounds, cepharosporolides C (10) E (11) and F (12) 2-carboxymethyl-4-(3-hydroxybutyl) furan (13) cordycepin (14) from fungus *Cordyceps militaris* BCC 2816. The antimalarial activity of 7-10 and 14 against *P. falciparum* K1 was estimated. Only compound 14 showed noteworthy effect with IC_{50} value of 4.5 µg/mL (10).

Another class of macrolides resorcylic acid lactones paecilomycins was also reported for antimalarial activities. Six new paecilomycins A-F (15-20) and five acknowledged aigilomycin B (21), zeanol (22), aigialomycin D (23), aigialomycin F (24) and aigialospirol were secluded from *Paecilomyces* sp. SC0924. Compounds 19 and 20 showed significant antimalarial activity in contradiction with *P. falciparum* line 3D7 with IC_{50} values of 20.0 and 10.9 nM, respectively, while rest of the compounds showed moderate activity (11). One year later, Xu and his colleagues isolated three paecilomycins G-I (26-28) from culture broth of the same fungus (12).

Three novel caprolactams (cyclic amide of caproic acid) named as pestalactams A–C (29-31),

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**Figure 1c. Fungal macrolides with antimalarial activity (15-28)**
were purified from *Pestalotiopsis* sp., an endophytic fungus of *Melaleuca quinquenervia*. Pestalactams A (29) and B (31) showed moderate activity against chloroquine-sensitive (IC₅₀ 16.2 and 20.7 µM, respectively) and chloroquine-resistant (IC₅₀ 41.3 and 36.3 µM, respectively) cell lines of *P. falciparum* (13).

**Piperazine derivatives**

Piperazines are six membered ring containing organic compounds possessing diverse pharmacological activities (antifungal, antibacterial, anticancer and antituberculosis) because of piperazine functional group. Tanaka, Hatabu and their colleagues reported that fungal metabolite gliotoxin (32) is an epipolythiodioxopiperazine toxin exhibiting significant plasmodicidal activity (8, 14). Gliotoxin is a sulfur containing mycotoxin produced by several species of fungi (15). In another study two ketopiperazines janoxepin (33) and brevicompanine B (34) were separated from *Aspergillus janus*. Janoxepin was a unique oxepin derivative possessing a rare D-leucine integrated. Both compounds showed antimalarial activity against *P. falciparum* 3D7 (IC₅₀ values of 28 and 35 mg/mL, respectively) (16).

**Terpene metabolites**

Terpenes are a huge class of naturally occurring hydrocarbons consisting of multiple isoprene units. Different classes of terpenic compounds include diterpenes, triterpenes, sesquiterpenes, steroidal lanosterol have been reported from various organisms such as plants and microorganism.

Tanaka and his colleagues reported a sesquiterpene antibiotic heptilidic acid (35) from fungi which showed antimalarial activities (8). Five trichothecenes (36-40) were isolated from *Myrothecium verrucaria* BCC 112. Trichothecenes are sesquiterpene mycotoxins produced by various fungal species possessing 12, 13-epoxy ring in their structure critical for biological potential. These compounds showed significant antimalarial activities with EC₅₀ values ranging from 0.06 to 0.90 ng/µL against *P. falciparium* (17). Later on, two more trichothecenes (41-42) compounds were also reported from Mangrove endophytic fungi. Compounds 41 and 42 showed potent antimalarial activity with IC₅₀ value below 20 nM (18). Isaka and his colleagues in 2000 reported two unique sesquiterpenes: (+)-phomenone (43) and (+)-phaseolinone (44) from *Xylaria* sp. BCC 1067. Both compounds showed promising antimalarial action with EC₅₀ values of 0.50 and 0.32 µg/mL, respectively (19).
In 2002, fungus *Drechslera dematioidea* was secluded from the interior tissue of the marine red alga *Liagora viscida* (20). Ten new sesquiterpenoids, specifically drechslerines A–G (47–52), isosativenetriol (45), sativene epoxide (54) and 9-hydroxyhelminthosporol (53). However, drechslerins E (50) and G (52) and helminthosporol (53) were isolated from *Drechslera dematioidea*. These compounds exhibited antimalarial activity against *P. falciparum*. Marine fungus *Halorosellinia oceanica* BCC 5149 from Thailand, produced an ophiobolane sesterterpene, halorosellinic acid (54), which showed antimalarial activity (IC<sub>50</sub> = 13 µg/mL) (21). Later on, further investigation of the same fungus led to isolation of another new ophiobolane sesterterpene, 17-dehydroxyhalorosellinic acid (56) (22).

Rukachaisirikul and his colleagues isolated two new sesquiterpenes, connatusins A&B (57, 58) and six known compounds (59–64) from the fungus *Lentinus connatus* BCC 8996. Compounds (61) panepoxydone, (62) panepoxydione and (64) dihydrohypnophilin exhibited significant antimalarial activities with IC<sub>50</sub> values of 3.4, 2.1 and 3.1 µg/mL, respectively (10). Chromatographic investigation of the extract of *Ganoderma lucidum* in ethyl acetate yielded seven triterpnes lanostanes (65–71) including three new (66, 67, 71) (Fig. 3c). These lanostanes exhibited moderate in vitro antiplasmodial activity with IC<sub>50</sub> values of 6 to 20 µM (23).
Antimalarial activity of spirodihydrobenzofuran terpenes was stated for the first time by Sawadjoon and his colleagues. They isolated two spirodihydrobenzofuran terpenic compounds (72) and 73 isolated from the fungus Stachybotrys nephrospora. Both compound showed antiplasmodial activity with IC₅₀ values of 0.85 and 0.15 µg/mL, respectively (24). Dichloromethane extract of an endophytic fungus Chalara alabamensis, secluded from Asterogyne martiana collected in Costa Rica displayed effective antimalarial activity against P. falciparum, with an EC₅₀ value of 24 µg/mL. Further fractionation led to isolation of viridiol 74 (Fig. 3d), which showed EC₅₀ value of 1.2 µg/mL against P. falciparum (25). Isaka and his colleagues reported five novel terpenoids sterostreins A-E (75, 76, 77a/77b, 78, and 79) isolated from mushroom Stereum ostrea BCC 22955. Sterostrein A unveiled potent antimalarial activity (IC₅₀ 2.3 µg/mL) against P. falciparum (26, 27).

Peptides

Peptides are biologically occurring small chains of amino acids linked through peptide bonds. Peptides can act as selective signaling molecules and are central in human physiology. Owing to their striking pharmacological profile signify them as an outstanding starting point for novel design of therapeutics. Nilanonta and his colleagues reported two cyclodepsipeptides from Paecilomyces tenuipes BCC 1614 (Fig. 4). Depsipeptide is a molecule that has both peptide and ester linkages in proximity and playing a vital role in early preclinical therapeutic discovery processes. Both compounds beauvericin (80) and beauvericin A (81) unveiled adequate antiplasmodial activities against K1 strain of P. falciparum with EC₅₀ values of 1.60 and 12.0 µg/mL, respectively (28).

Isaka and his colleagues also reported cyclohexadepsipeptides pullularins A-D (82-85) from Aureobasidium pullulans, isolated from a leaf of

Figure 3b. Fungal terpene metabolites with antimalarial activity
Culophyllum sp. from Thailand. Pullularin A and B exhibited antimalarial activities with IC<sub>50</sub> values of 3.6 and 3.3 µg/mL, respectively (29). In another study Isaka and his group also reported isolation of a new cyclohexadepsipeptide paecilodepsipeptide A (86) and its two linear analogues paecilodepsipeptides B and C (87, 88) from Paecilomyces cinnamomeus BCC 9616. Paecilodepsipeptide A (1) showed activity in contradiction of P. falciparum K1 with an IC<sub>50</sub> value of 4.9 µM (29) (Fig. 4).

Tetramic acid derivatives

Tetramic acid derivatives are nitrogenous heterocyclic compounds containing pyrrolidine-2,4-dione. They are important structural units in many natural products acquired from earthly and marine sources (30). They show a broad spectrum of biological actions which includes: cytotoxic, antibiotic, antifungal, enzyme inhibitory and antiviral activities (31).

Osterhage and his coworkers reported two new unusual tetramic acid derivatives ascosalipyrrolidinones A (89) and B (90) as well as the new pyrone ascosalipyrone (91) from marine endophytic fungus Ascochyta salicorniae (Fig. 5). Ascosalipyrrolidinone A (89) showed antiplasmodial action in contradiction of P. falciparum with IC<sub>50</sub> value of 736 ng/mL (32).
Xanthones

Xanthones are polyphenolic compounds that exhibit extensive biological and pharmacological activities. Isaka and his colleagues reported isolation of two novel xanthone dimers, phomoxanthones A and B (92, 93) along with compound 94 (deacetyl analogue of 92) from an endophytic fungus *Phomopsis* sp. BCC 1323 (33). Compounds 92 and 93 demonstrated notable antimalarial actions in contradiction to *P. falciparum* K1 with IC_{50} values of 0.11 and 0.33 µg/mL, respectively. In another study, ascherxanthone A (95), a novel tetrahydroxanthone dimer, was isolated from fungus *Aschersonia* sp. BCC 8401 (Fig. 6). Ascherxanthone A exhibited activity against *P. falciparum* K1 with an IC_{50} value of 0.20 µg/mL (34). Pontius and his group evaluated antiparasitic potential of extract of marine *Chaetomium* fungus. They also reported isolation and purification of three new xanthones, chaetoxanthones A-C (96-98) (35). Compound 97 exposed selective activity against *P. falciparum* with IC_{50} of 0.5 µg/mL.

Pyridone

Pyridone derivatives contain amide and keto groups in it and are well known for their biological activities. Four N-hydroxy- and N-methoxy-2-pyridone compounds cordypyridones A-D (99-102) were purified from *Cordyceps nipponica* BCC 1389 (Fig. 7). Cordypyridones A and B exhibited compelling antimalarial action with IC_{50} values of 0.066 and 0.037 µg/mL, respectively (36).

Coumarins

Coumarin is a natural organic compound belonging to benzopyrones possessing biological activities (37). Kongsaeree and his coworkers isolated three novel antimalarial dihydroisocoumarin...
derivatives (103-105) from an endophytic fungus, *Geotrichum* sp. (38). Compounds 103 and 105 showed antimalarial action in contradiction of *P. falciparum* with IC₅₀ values of 4.7 and 2.6 µg/mL, respectively. In 2008, Sappapan and his coworkers reported isolation of a new analogue of monocerin, 11-hydroxymonocerin (107) along with known 12-

Quinone derivative

Quinones are oxidized derivatives of aromatic compounds. Quinones possess many pharmacological activities such as anti-tumor, purgative, antimicrobial and antiparasitic. A quinone derivative (7-hydroxy-8-methoxy-3,6-dimethylidibenzofuran-1,4-dione) (109) and an organohalogen natural product (2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione) (110) were isolated from the organic extract of *Xylaria* sp. PBR-30 an endophytic fungus isolated from *Sandoricum koetjape* (Fig. 9). Compounds 109 and 110 showed antimalarial against *P. falciparum* K1 strain with IC₅₀ values of 1.84 and 6.68 µM, respectively (40).

Hydronaphthalenone derivatives

Sommart and his group reported isolation of three new hydronaphthalenone derivatives (110-112) from the unidentified PSU-N24 endophytic fungus.
fungus of *Garcinia nigrolineata* (Fig. 10). Compound 112 showed antimalarial action with the IC$_{50}$ value of 7.94 µg/mL (41).

**Tryptophan derivatives**

Codinaeopsin 113 a newfangled tryptophan-polyketide hybrid which comprises an unusual heterocyclic unit linking indole and decalin fragments, was aquired from the crude extract *Codinaeopsis gonytrichoides* an endophytic fungus isolated from *Vochysia guatemalensis* obtained from Costa Rica (Fig. 11). Codinaeopsin showed activity against 3D7 strain of *P. falciparum* with an IC$_{50}$ value of 2.3 µg/mL (42).

![Figure 5. Fungal tetramic acid metabolites with antimalarial activity](image)

![Figure 6. Fungal xanthone metabolites with antimalarial activity](image)
Figure 7. Fungal pyridone metabolites with antimalarial activity

Figure 8. Fungal coumarins metabolites with antimalarial activity

Figure 9. Fungal quinones metabolites with antimalarial activity

Figure 10. Fungal hydronaphthalenone metabolites with antimalarial activity

110 $R_1 = R_2 = R_3 = R_5 = H, R_4 = \alpha H, R_6 = OH$
111 $R_1 = R_2 = R_3 = R_5 = H, R_4 = \beta H, R_6 = OH$
112 $R_1 = OH, R_2 = R_3 = R_5 = H, R_4 = \alpha H, R_6 = OH$
Tropane derivatives

Tropane is a nitrogenous compound which is bicyclic amine containing piperidine and piperidine rings. Tropane is the same structural part of all tropane alkaloids (43). Tropane derivatives are antiemetics, antispasmodics, anesthetics, and bronchodilators (44). Pycnidione (114), bistropolone a vastly oxygenated compound obtained from marine fungi showed antimalarial activity in contradiction of three strains of *P. falciparum* FCR3F86, W2 and D6 (IC₅₀ = 0.28, 0.37 and 0.75 µM, respectively) (45). Iwatsuki and his coworkers also reported five (115-119) tropolone compounds, isolated from *Penicillium* sp. FKI-4410. Compounds (115, 116) were named as puberulic acid and stipitatic acid.
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While three compounds were new analogs of puberulic acid, named as viticolins A–C (117-119). Among them, puberulic acid (115) unveiled effective antimalarial activity in contradiction of chloroquine resistant \textit{P. falciparum} strain with IC$_{50}$ value of 0.01 µg/mL while other compounds did not showed significant activity (46).

Benzaldehyde derivatives

Four benzaldehyde derivatives (E)-2-(hept-1-enyl)-3-(hydroxymethyl)-5-(3-methylbut-2-enyl)benzene-1,4-diol (120), flavoglaucin (121), tetrahydroauroglaucin (122), auroglaucin (124), and 2-(2',3-epoxy-1',3'-heptadienyl)-6-hydroxy-5-(3-methyl-2-butenyl)benzaldehyde (125) demonstrated restrained antimalarial actions with IC$_{50}$ values ranging from 1.1 to 3.0 µg/mL when tested in contradiction of chloroquine-sensitive (D6, Sierra Leone) and chloroquine-resistant (W2, Indo China) strains of \textit{P. falciparum}. Antimalarial activity of auroglaucin (124) was stronger than other compounds (IC$_{50}$ 1.8 and 1.1 µg/mL against D6 and W2, respectively) (47).

Cytochalasins

Cytochalasins are considered an important class of cytotoxic fungal metabolites. Cytochalasins possess various biological actions. Calcul and his colleagues reported cytochalasins (128-135). All cytochalasins were found energetic in contradiction of \textit{P. falciparum} strain with IC$_{50}$ values between 20-136 nM (18).

Polyamine alkaloid

Bioassay-guided fractionation of extract of fungus \textit{Ramaria subaurantiaca} led to isolation of known polyamine alkaloid, pistillarin (136) which exhibited significant antimalarial activity with IC$_{50}$ of 1.9 µM (48).

Extracts with antimalarial activity

Wiyakrutta and his colleagues reported biological actions of extracts of endophytic fungi
secluded from Thai medicinal plants. Extracts from 92 endophytes were experienced for antimalarial activity in contradiction of \textit{P. falciparum} and extracts of six of the extracts showed significant activity with IC$_{50}$ values ranging between 1.2ñ9.1 µg/mL (48). Singh and Parkash in 2005 reported antimalarial activity of culture filtrate of \textit{Beauveria bassiana} fungus. Govindarajan and his group in 2005 studied antimalarial activity of culture filtrate of various fungal species (49). Antiplasmodial potential of aqueous extract of a fungus, \textit{Chlonophyllum molybdites} was determined in Swiss albino mice at a dose of 200 mg/kg/day given orally. Results showed that extract had antimalarial potential (50). Oluba and his coworkers reported that all biochemical derangements that characterized \textit{P. berghei} malarial infection in mice were restored to normal/near normal levels following treatment with aqueous extract of the fruiting bodies of \textit{G. lucidum} (51). In another study, Higginbotham and his colleagues reported isolation of 2700 fungal endophytes and almost 16.9% of fungal genotypes selected in their work were extremely active in contradiction of \textit{P. falciparum} (52). Eighty four different fungal extracts were studied for their antiplasmodial activity in contradiction of blood stage \textit{P. falciparum} in human red blood cell culture. Results publicized that fungal endophytes belonging to diverse genera showed antiplasmodial action such as \textit{Fusarium} sp. (IC$_{50}$: 1.94 µg/mL) and \textit{Nigrospora} sp. (IC$_{50}$: 2.88 µg/mL) (53).

\textbf{Future perspectives and conclusion}

New bioactive metabolites are a need of time due to ever increasing dilemma of microbial resistance to current therapeutic and control agents along with emergence of new life threatening diseases. These problems have pushed scientists to look in unconventional sources like endophytes for the new novel compounds. The fungus capacity to synthesize a variety of new novel bioactive metabolites forced researchers to explore these avenues.

\textbf{REFERENCES}


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Dutasteride (N-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide) is an active pharmaceutical ingredient (API) which inhibits the conversion of testosterone to dihydrotestosterone. As a 5-reductase inhibitor dutasteride is useful for the treatment of benign prostatic hyperplasia (BPH) and prostate cancer. Because of a large variety of solvents and reagents used in the synthesis, it was necessary to develop new, sensitive and selective gas chromatography (GC) methods. The optimization of the methods consisted in selecting different types of the sample injection and detection as well as the optimization of experimental conditions that allowed to meet the appropriate range of permissible limits and suitable detection limits (LOD) of compounds. Significant differences in the volatility of these compounds forced the division into volatile solvents (methanol, acetonitrile, dichloromethane, ethyl acetate, heptane and toluene) analyzed with the use of the gas chromatography method with headspace (GC-HS) and less volatile compounds (pyridine, dimethylformamide, 1,4-dioxane, acetic acid, ethylene glycol, 4-dimethylaminopyridine) which were analyzed using gas chromatography with direct injection (GC-FID). Benzene, carbon tetrachloride and 1,2-dichloroethane are potential contaminants of toluene and dichloromethane, thus the control of these solvents was a limit test procedure. Due to the low permissible limits for benzene (2 µg/mL), carbon tetrachloride (4 µg/mL) and 1,2-dichloroethane (5 µg/mL) it was necessary to use gas chromatography with a mass spectrometry detector (GC-MS). All three new methods were validated according to the requirements of the ICH (International Conference on Harmonization) validation guideline Q2R1 and the Q3C guideline for residual solvents. Specificity, precision, accuracy, linearity, limits of detection and quantitation as well as robustness were determined and the results meeting the acceptance criteria were obtained.

Keywords: GC method, GC-HS method, GC-MS method, validation, dutasteride, residual solvents

Dutasteride (N-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide) is an active pharmaceutical ingredient (API) which inhibits both 1 and 2 type isozymes of 5α-reductase, the enzyme responsible for converting testosterone to dihydrotestosterone in the prostate and other tissues. Dihydrotestosterone is the primary cause of prostate growth and has been proven to play a key role in the development and progression of benign prostatic hyperplasia and prostate cancer (1-4).

The process of synthesis that was used to manufacture dutasteride is described in the literature (5, 6). According to the synthesis route, many organic chemical compounds were used which can be classified as residual solvents in conformity of the Q3C(R5) guideline by ICH (International Conference on Harmonization). In this guideline all solvents are classified into three classes with different acceptable limits, mainly based on their toxicity.

Residual solvents are defined as organic volatile chemical compounds that are used throughout the process of pharmaceutical substance manufacturing. The residues of solvents remain in the final product despite various attempts to remove them. It is essential to estimate and control the concentration of every individual chemical compound. Gas chromatography based methods are recommended to quantify and quantitate residual solvents.
indicated in the ICH and pharmacopeial guidelines. The solvents and volatile reagents used during the manufacturing process of dutasteride active pharmaceutical ingredient are: methanol, acetonitrile, dichloromethane, ethyl acetate, heptane, toluene (as volatile chemical compounds) and pyridine, dimethylformamide, 1,4-dioxane, acetic acid, ethylene glycol, 4-dimethylaminopyridine (as less volatile compounds). The separation of the chemical compounds into two groups was induced by the need to use two injection methods: direct liquid sample injection and gaseous sample injection with the headspace system.

Three new gas chromatography methods were developed to quantitate the residual solvents and volatile reagents. The direct injection method was developed for less volatile organic compounds to ensure the best limits of detection and quantification. The headspace technique was used to inject gaseous samples and to analyze volatile compounds. The third method (limit test according to the Q2(R1) guideline by ICH) was developed for benzene, carbon tetrachloride and 1,2-dichloroethane as potential contaminants of toluene and dichloromethane. The use of gas chromatography hyphenated with a mass spectrometric detector was necessary to acquire results for very low concentrations of the analytes (specification limit for benzene 2 µg/mL, carbon tetrachloride 4 µg/mL and 1,2-dichloroethane 5 µg/mL). All three methods were validated and the results meeting the acceptance criteria were obtained.

These are the first methods reported in the literature for the organic volatile impurities determination in dutasteride. The monograph related to dutasteride is presented in the European Pharmacopoeia (EP) but does not contain GC methods for the volatile compounds determination. The development of the analytical methods and their validation are presented in this article.

EXPERIMENTAL

Chemicals and reagents
The active substance dutasteride was synthesized in Pharmaceutical Research Institute (Warsaw, Poland). The reagent 4-dimethylaminopyridine (DMAP) was obtained from Aldrich (Germany). The solvents and diluents were purchased from commercial suppliers: dichloromethane (DCM), pyridine, N,N-dimethylformamide (DMF), 1,2-dichloroethane, ethanol from Merck (Germany), 1,4-dioxane, ethylene glycol, heptane, methanol, ethyl acetate, acetonitrile, toluene, carbon tetrachloride from POCH (Poland), acetic acid from Sigma Aldrich (Germany), benzene from Fluka and N,N-dimethylacetamide (DMA) from J.T.Baker (Germany).

Methods
Because of a large variety of solvents and reagents used in the synthesis, it was necessary to develop three gas chromatography methods. Different detection level and significant differences in the volatility of these compounds forced the division into less volatile compounds (pyridine, N,N-dimethylformamide, 1,4-dioxane, acetic acid, ethylene glycol and 4-dimethylaminopyridine) analyzed with the use of gas chromatography with direct injection (method I – GC-FID) and volatile solvents (methanol, acetonitrile, dichloromethane, ethyl acetate, heptane and toluene) which were analyzed using the gas chromatography method with headspace (method II – GC-HS). Benzene, carbon tetrachloride and 1,2-dichloroethane were analyzed with the use of gas chromatography with mass spectrum detection (method III – GC-MS) because of the low specification limits for the solvents.

Method I (GC-FID)
Equipment and chromatographic conditions
The method was performed on a Shimadzu GC-2010 gas chromatograph with a flame ionization detector interacted with an auto-sampler AOC20i. A DB-WAX column (phase composition: polyethylene glycol) film thickness 0.5 µm, 60 m long and 0.32 mm ID, from Agilent Technologies was used. The following oven temperature program was used: the initial temperature of 80°C was raised at the rate of 5°C/min to the temperature of 180°C, then increased at 30°C/min to 240°C and held at this level for 9 min. The injection port temperature was 220°C and the detector temperature was 260°C. Nitrogen was used as the carrier gas at 100 kPa, split 5 : 1, 1 µL of the solution was injected into the gas chromatograph.

Sample and standard solutions preparation
The sample solution was prepared by dissolving an appropriate amount of dutasteride in DCM. The standard solution was prepared by dissolving appropriate amounts of determined solvents and reagents in DCM and subsequent dilution to reach 380 µg/mL of 1,4-dioxane, 5000 µg/mL of acetic acid, 620 µg/mL of ethylene glycol, 200 µg/mL of pyridine, 880 µg/mL of DMF, 1500 µg/mL of DMAP with respect to the sample preparation (100% of the permissible limit).
Method II (GC-HS)

**Equipment and chromatographic conditions**

The method was performed on a Perkin Elmer CLARUS 500 gas chromatograph with a flame ionization detector interfaced with a Perkin Elmer headspace auto-sampler TURBOMATRIX 40. Chromatographic separation was performed on a DB-624 column (phase composition: 6% cyanopropylphenyl – 94% dimethylpolysiloxane), 60 m long, 0.32 mm ID, 1.8 µm film thickness, from Agilent Technologies.

The following oven temperature program was used: the initial temperature of 60°C was raised at the rate of 2°C/min to the temperature of 75°C, then increased at 5°C/min to 120°C, next increased at 40°C/min to 240°C and held at this level for 3 min. The injection port temperature was 240°C and the detector temperature was 260°C. Nitrogen was used as the carrier gas at 100 kPa, split 5 : 1, attenuation 5. The vial oven temperature was set at 100°C for 30 min. The needle temperature was 110°C, the transfer line was 120°C, inject: 0.05 min.

**Sample and standard solutions preparation**

The sample solution was prepared by dissolving an appropriate amount of dutasteride in DMA. The standard solution was prepared by dissolving appropriate amounts of determined residual solvents in DMA and subsequent dilution to reach 3 µg/mL of benzene, 4 µg/mL carbon tetrachloride and 5 µg/mL 1,2-dichloroethane with respect to the sample preparation (100% of the permissible limit).

Method III – GC-MS

**Equipment and chromatographic conditions**

The method was conducted on a Shimadzu GC-2010 Plus with an MS detector GCMS-QP2010Ultra, both from Shimadzu. A DB-5MS column (phase composition: 5% phenyl-methylpolysiloxane – 95% phenyl arylene polymer) film thickness 0.25 µm, 30 m long and 0.25 mm ID, from Agilent Technologies was used.

The oven temperature program was as follows: the initial temperature of 40°C was ramped up at the rate of 5°C/min to 60°C, next ramped up at the rate of 40°C/min to 280°C and maintained for 5.5 min. The injection port temperature was 280°C and the detector temperature was 280°C. Nitrogen was used as the carrier gas at 60 kPa, split 4 : 1, 2 µL of the solution was injected into the gas chromatograph. Characteristic ions: benzene m/z 78, carbon tetrachloride m/z 117 and 1,2-dichloroethane m/z 62.

**Sample and standard solutions preparation**

The sample solution was prepared by dissolving an appropriate amount of dutasteride in DMA. The standard solution was prepared by dissolving appropriate amounts of determined compounds in DMA and subsequent dilution to reach 2 µg/mL of benzene, 4 µg/mL carbon tetrachloride and 5 µg/mL 1,2-dichloroethane with respect to the sample preparation (100% of the permissible limit).

RESULTS AND DISCUSSION

**Methods development**

During the development of the GC method with direct injection for the determination of pyridine, DMF, 1,4-dioxane, acetic acid, ethylene glycol and DMAP the following columns were tested: DB-624, HP PLOT/Q, DB-5, DB-CAM. On most of these columns wide and tailing peaks with too low sensitivity were obtained, or sufficient separation from the rest of the solvents were not obtained. Finally, the column DB-WAX was selected, and on this column good separation of the analytes and adequate method sensitivity were obtained.

Appropriate diluents without interfering peaks at the retention times of the determined solvents were chosen for the preparation of the sample. In the course of the accuracy study of the developed methods it has been showed that the matrix does not affect the results of the solvents determination.

**Methods validation**

All the described methods were validated according to the requirements of the ICH (International Conference on Harmonization) validation guideline Q2R1 and the Q3C guideline for residual solvents. The validation of the methods (quantitative test procedure) included the examination of specificity, linearity, accuracy, repeatability, intermediate precision, system precision, robustness as well as quantitation and detection limits.

**Specificity**

**Specificity of method I**

The specificity of the method was evaluated by injecting the solution containing all solvents and reagents from the synthesis route and their potential contaminants at 100% of the permissible limit: 1,4-dioxane (380 µg/mL), pyridine (200 µg/mL), DMF (880 µg/mL), acetic acid (5000 µg/mL), ethylene glycol (620 µg/mL), DMAP (1500 µg/mL), methanol (3000 µg/mL), acetonitrile (410 µg/mL), ethyl acetate (5000 µg/mL), heptane (5000 µg/mL), toluene (890 µg/mL), benzene (2 µg/mL), carbon...
tetrachloride (4 µg/mL), 1,2-dichloroethane (5 µg/mL). In this method benzene and 1,2-dichloroethane were not found. The retention time for all compounds was determined by analyzing individual solvent solutions. The peaks of methanol, ethyl acetate and carbon tetrachloride were in the peak of DCM (diluent of the sample). All peaks in the chromatogram of the specificity solution were completely separated from the analytes (R_s = 1.5), R_s: heptane/DCM ñ 3.70, DCM + methanol + ethyl acetate + carbon tetrachloride /benzene ñ 5.25, acetonitrile/toluene ñ 4.88, toluene/1,4-dioxane ñ 4.16, 1,4-dioxane/pyridine ñ 23.43, pyridine/DMF ñ 35.37, DMF/acetic acid ñ 20.82, acetic acid/ethylene glycol ñ 44.52, ethylene glycol/DMAP ñ 27.72. Spiking the sample solution with the analytes did not cause the peaks to split and the retention times remained the same as for the corresponding peaks from the sample solution. The chromatogram of the specificity solution is presented in Figure 1A.

**Specificity of method II**

The specificity of the method was evaluated by injecting the solution containing all solvents and reagents from the synthesis route and their potential contaminants at 100% of the permissible limit: methanol (3000 µg/mL), acetonitrile (410 µg/mL), DCM (600 µg/mL), ethyl acetate (5000 µg/mL), heptane (5000 µg/mL), toluene (890 µg/mL), 1,4-dioxane (380 µg/mL), pyridine (200 µg/mL), DMF (880 µg/mL), acetic acid (5000 µg/mL), ethylene
Development and validation of gas chromatography methods for...

glycol (620 µg/mL), DMAP (1500 µg/mL), benzene (2 µg/mL), carbon tetrachloride (4 µg/mL), 1,2-dichloroethane (5 µg/mL). In this method acetic acid, ethylene glycol, DMF, benzene, carbon tetrachloride and DMAP were not found, the retention time of 1,2-dichloroethane was the same as the retention time of the heptane impurity. The retention time for all compounds was determined by analyzing individual solvent solutions. Ethanol was used to dissolve heptane in order to prepare the standard solution. The selectivity showed no co-elution of ethanol and the solvents determined by this method. All peaks in the chromatogram of the specificity solution were completely separated (Rs = 1.5). Spiking the sample solution with the analytes did not cause the peaks to split and the retention times remained the same as for the corresponding peaks from the sample solution. The chromatogram of the specificity solution is presented in Figure 1B.

Specificity of method III

The specificity was examined using a standard solution (a solution consisting of the analyzed solvents at 100% of the specification limit: benzene (2 µg/mL), carbon tetrachloride (4 µg/mL), 1,2-dichloroethane (5 µg/mL). The use of the mass detector in gas chromatography ensures the specificity of the method by the extraction of a specific m/z (ion mass) chromatogram from a total ions chromatogram for all the solvents. In the said method characteristic signals were chosen from the mass spectrum of solvents. Characteristic ions were selected for: benzene mean ion – m/z 78 and comparative ion – m/z 51, carbon tetrachloride mean ion – m/z 117 and comparative ion – m/z 119 and 1,2-dichloroethane mean ion – m/z 62 and comparative ion – m/z 49.

Peaks of benzene, carbon tetrachloride and 1,2-dichloroethane were not observed in the chromatogram of the test solution. Spiking the sample with the analyte did not cause a peak to split; no additional peaks appeared apart from that one of the analyte. The matrix did not affect the analytical results. The chromatogram of the standard solution is presented in Figure 1C.

Linearity

It was checked that the response of the detector is linear to the concentration of the analyte for all the
compounds. The linearity was evaluated by the linear regression analysis to calculate the slope, y-intercept, and correlation coefficient ($R^2$). An additional restriction was that Student t-test should be passed ($t_{cr} = 2.78$, $t_{cr} > |t_{b}| < t_{cr}$). The linearity of the methods was evaluated by analyzing 7 solutions ranging in the concentration of the analytes from about 10% (or LOQ) to 120% of the specified limit (methods I and II) and from about 30% to 120% of the specified limit (method III). All concentrations were prepared in triplicate and the average was reported. The methods were linear within a wide range for the solvents included in the validation. The acceptance criteria were confirmed. The results are presented in Figure 2 (method I) and in Tables 1, 2 (methods II and III).

**Range**

The range concentrations from 10% to 120% of the specification limit: pyridine 20–240 µg/mL, DMF 88–1056 µg/mL, ethylene glycol 62–744 µg/mL, 1,4-dioxane 38–456 µg/mL, methanol 300–3600 µg/mL, acetonitrile 41–492 µg/mL, DCM 60–720 µg/mL, ethyl acetate 500–6000 µg/mL, heptane 500–6000 µg/mL, toluene 89–1068 µg/mL; the range concentrations from 30% to 120% of the specification limit: benzene 0.6–2.4 µg/mL, carbon tetrachloride 1.2–4.8 µg/mL, 1,2-dichloroethane 1.5–6.0 µg/mL, DMAP 500–1800 µg/mL; the range concentrations from 50% to 120% of the specification limit: acetic acid 2500–6000 µg/mL. The results of the analyses confirmed that the methods in these ranges are precise, linear and accurate.

![Figure 3. Results of the accuracy determination (method I)](image.png)
Accuracy

The accuracy of the analytical methods was reported as the percentage of the compound added to the sample solution in comparison to the true value. The accuracy of the method was established by assaying 9-12 sample solutions: triplicate independent preparations for 3-4 concentrations (the sample solutions spiked with the analytes at about 10%, 30%, 50%, 100%, 120% of the specification limit). The recovery for all the solutions had to be within

Table 1. Validation results of the method II (1 - methanol, 2 - ACN, 3 - DCM, 4 - ethyl acetate, 5 - heptane, 6 - toluene).

<table>
<thead>
<tr>
<th>Test</th>
<th>Acceptance criteria</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity</strong></td>
<td>Correlation coefficient (R²)</td>
<td>&gt; 0.990</td>
<td>0.999</td>
<td>0.999</td>
<td>0.998</td>
<td>0.998</td>
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<tr>
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<td>y-Intercept (b)</td>
<td>-422.16</td>
<td>52.85</td>
<td>117.15</td>
<td>2401.50</td>
<td>8215.74</td>
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<td></td>
<td>Slope (a)</td>
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<td>41.75</td>
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<td>58.47</td>
<td>344.89</td>
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<td>65.98</td>
<td>42.73</td>
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<td></td>
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<td>0.38</td>
<td>0.26</td>
<td>0.50</td>
<td>0.44</td>
<td>0.26</td>
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<tr>
<td><strong>Accuracy</strong></td>
<td>Mean recovery [%]</td>
<td>80-120%</td>
<td>93.04</td>
<td>99.02</td>
<td>97.33</td>
<td>102.15</td>
<td>99.76</td>
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<td></td>
<td>RSD [%]</td>
<td>15%</td>
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<td>2.05</td>
<td>2.13</td>
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<td></td>
<td></td>
<td>15%</td>
<td>3.19</td>
<td>2.02</td>
<td>2.04</td>
<td>4.85</td>
<td>5.12</td>
</tr>
<tr>
<td></td>
<td>RSD [%]</td>
<td>15%</td>
<td>4.30</td>
<td>3.03</td>
<td>5.21</td>
<td>4.90</td>
<td>5.21</td>
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<tr>
<td></td>
<td>(Solutions 100%)</td>
<td>15%</td>
<td>1.98</td>
<td>1.97</td>
<td>6.73</td>
<td>4.95</td>
<td>2.97</td>
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<tr>
<td><strong>Repeatability</strong></td>
<td>RSD [%]</td>
<td>15%</td>
<td>1.43</td>
<td>2.81</td>
<td>1.36</td>
<td>1.11</td>
<td>1.08</td>
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<td>(Solutions 100%)</td>
<td>10%</td>
<td>0.45</td>
<td>0.83</td>
<td>2.67</td>
<td>1.02</td>
<td>1.94</td>
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<tr>
<td></td>
<td>RSD [%]</td>
<td>15%</td>
<td>2.08</td>
<td>2.97</td>
<td>5.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Solutions 100%)</td>
<td>15%</td>
<td>2.76</td>
<td>3.12</td>
<td>4.30</td>
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<tr>
<td><strong>Intermediate</strong></td>
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<td>4.94</td>
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<td><strong>System</strong></td>
<td>RSD [%]</td>
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<td>3.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>precision</td>
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Table 2. Validation results of the method III.

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<th>Carbon tetrachloride</th>
<th>1,2-Dichloroethane</th>
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<td></td>
<td>y-Intercept (b)</td>
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<td>17.22</td>
<td>-17.45</td>
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<td></td>
<td>Slope (a)</td>
<td>1976.8</td>
<td>845.8</td>
<td>388.7</td>
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<td></td>
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<td>126.5</td>
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<td></td>
<td></td>
<td>0.44</td>
<td>0.42</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>Mean recovery [%]</td>
<td>80-120%</td>
<td>96.90</td>
<td>101.15</td>
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<tr>
<td></td>
<td>RSD [%]</td>
<td>15%</td>
<td>1.71</td>
<td>4.02</td>
</tr>
<tr>
<td></td>
<td>(Solutions 30% + 120%)</td>
<td>15%</td>
<td>2.08</td>
<td>2.97</td>
</tr>
<tr>
<td></td>
<td>(Solutions 100%)</td>
<td>15%</td>
<td>2.76</td>
<td>3.12</td>
</tr>
<tr>
<td><strong>Repeatability</strong></td>
<td>RSD [%]</td>
<td>15%</td>
<td>2.44</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>(Solutions 100%)</td>
<td>10%</td>
<td>3.52</td>
<td>3.14</td>
</tr>
<tr>
<td><strong>Intermediate</strong></td>
<td>RSD [%]</td>
<td>10%</td>
<td>2.11</td>
<td>2.85</td>
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<tr>
<td><strong>precision</strong></td>
<td>(Solutions 30%)</td>
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</table>
the range from 80 to 120%. The results of the value of the recovery are presented in Figure 3 (method I) and in Tables 1, 2 (methods II and III). The acceptability criteria were fulfilled.

**Precision**

Precision of the analytical methods was considered at three levels: repeatability, intermediate precision and system precision. Precision of an analytical procedure is usually expressed as the relative standard deviation (RSD) of a series of measurements.

The repeatability was performed by measuring triplicate independent preparations for three concentrations – the sample solution spiked with the analytes at about 10% (or 30%, 50%), 100%, 120% of the specification limit and 6 independent solutions – the sample solution spiked with the analytes at about 100% of the specification limit, then the relative response (the relation of peak area to mass) was calculated. The intermediate precision was repeated on a different day by a different analyst by measuring 6 independent solutions – the sample solution spiked with the analytes at about 100% of the specification limit, then the relative response (the relation of peak area to mass) was calculated. The comparison of the repeatability and intermediate precision results was performed using the F-Snedecor test: \( F = F_{cr} (F_{cr} = 5.05); (n = 6) \) \( (\alpha = 0.05, f_1 = n_1 - 1, f_2 = n_2 - 1) \). The results of the parameter \( F \) for the solvents: 1,4-dioxane 1.60, pyridine 1.62, DMF 1.36, acetic acid 1.51, ethylene glycol 1.86, DMAP 1.45, methanol 0.25, acetonitrile 2.25, DCM 1.96, ethyl acetate 1.00, heptane 2.78, toluene 2.25, benzene 1.00, carbon tetrachloride 2.25, 1,2-dichloroethane 2.25.

The system precision was established by measuring the response of six replicate injections of the standard solution with the analytes at 100% of the specification limit and six replicate injections of the standard solution with the analytes at 10% or 30% of the specification limit. The results are expressed as the RSD and summarized in Figure 4 (method I) and in Tables 1, 2 (methods II and III). All the criteria were fulfilled.

**Limit of Quantitation (LOQ) and Limit of Detection (LOD)**

The limit of detection (LOD) and limit of quantification (LOQ) were estimated as signal-to-noise ratio. The LOQ and LOD were evaluated using the standard solutions containing known low concentrations of the solvents. The concentration which generated the peak height of the solvent at least 10 times as high as the noise’s height was established as the LOQ and the one at least 3 times as high as the noise’s height was stated as the LOD. The following LOD and LOQ values were obtained (with respect to the sample preparation): methanol – 9.0 µg/mL, 19 µg/mL, acetonitrile – 8.0 µg/mL, 25 µg/mL, DCM – 13.8 µg/mL, 40 µg/mL, ethyl acetate – 7.5 µg/mL, 68 µg/mL, heptane – 1.2 µg/mL, 4 µg/mL, toluene – 7.2 µg/mL, 24 µg/mL, pyridine – 2.4 µg/mL, 6.6 µg/mL, DMF – 6.0 µg/mL, 20 µg/mL, 1,4-dioxane – 3.3 µg/mL, 11 µg/mL, acetic acid – 22 µg/mL, 68 µg/mL, ethylene glycol – 17 µg/mL, 44 µg/mL, DMAP – 180 µg/mL, 500 µg/mL.

![Figure 4. Results of the precision determination (method I)](image-url)
benzene – 0.02 µg/mL, 0.03 µg/mL, carbon tetrachloride – 0.18 µg/mL, 0.44 µg/mL, 1,2-dichloroethane – 0.23 µg/mL, 0.83 µg/mL, respectively.

Robustness

The robustness of the methods was evaluated by injecting the specificity solutions to ensure the separation of all solvents from the synthesis route with the use of different chromatographic conditions. The following parameters were tested: column temperature ± 5°C, rate ± 1°C/min and carrier gas pressure ± 10%. The smallest resolution (Rs = 1.72) was obtained between pyridine and toluene at the rate 1°C/min. in method II. Changes in the analytical conditions did not influence the resolution significantly and the method was robust.

CONCLUSION

Sensitive, accurate and precise GC methods for the determination of residual solvents (their potential contaminants) and volatile reagents used in the manufacturing process of dutasteride were developed. Impurities are often not removed completely by practical manufacturing techniques and consequently their low levels are present in most pharmaceuticals.

Complete validations of the quantitative test procedures to control the presence of these compounds (methanol, acetonitrile, dichloromethane, ethyl acetate, heptane, toluene, pyridine, dimethylformamide, 1,4-dioxane, acetic acid, ethylene glycol, 4-dimethylaminopyridine, benzene, carbon tetrachloride and 1,2-dichloroethane) were performed. The methods turned out to be specific, accurate, linear and precise. The compounds were detected and quantified at a µg/mL level. The validation results clearly demonstrate that the analytical procedures are suitable for their intended purpose.

The developed GC methods were successfully used for the routine quality control of different batches of dutasteride under GMP rules. All analyzed batches were declared to be in compliance with the in-house specifications of residual solvents.

Acknowledgments

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We wish to thank Ms. Joanna Zagrodzka, Ph.D. and the employees of the Chemistry Department for manufacturing and delivering the samples for testing and for their support in this study.

REFERENCES


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Polish Pharmaceutical Society

Isoniazid (INH) (Fig. 1), chemically known as pyridine-4-carboxylic acid hydrazide, is an antitubercular drug now widely used together with rifampicin and streptomycin for the chemotherapy of tuberculosis. This has prompted many investigators to devise methods for its determination in its pure form as well as in its tablet form.

The drug is official in Indian Pharmacopoeia (IP) (1), British Pharmacopoeia (BP) (2) and United State Pharmacopeia (USP) (3). IP and BP describe titration of the drug with potassium bromate in the presence of potassium bromide using methyl red indicator. USP describes HPLC method using L1 column (4.6 mm × 25 cm) and a mobile phase consisting of methanol : water (40 : 60) (pH adjusted to 2.5 with H₂SO₄) with a flow rate of 1.5 mL/min and UV-detection at 254 nm. Apart from the above official methods, a number of methods based on several techniques are found in the literature for INH and include: titrimetry (4-7), voltammetry (8-13), ion selective electrode-potentiometry (14-19), amperometry (20, 21), spectrofluorimetry (22, 23), chemiluminescence spectrometry (24-33), high performance liquid chromatography (HPLC) (34-37), gas chromatography (GC) (38-40), LC/LC-MS (41) and capillary electrophoresis (42-44).

Visible spectrophotometry is by far the most widely used technique for the assay of INH. Methods based on a variety of reaction schemes such as, nucleophilic substitution (45), condensation (46-49), charge-transfer and ion-pair (50), derivatization (51-54), diazo-coupling (55, 56), oxidative coupling (57, 58), complex formation (59-61) and redox followed by complexation (62-65). These methods suffer from the disadvantages such as drastic experimental conditions, use of organic solvent, longer standing time, poor sensitivity, narrow linear range, etc.

KMnO₄ has been a very useful reagent for the spectrophotometric assay of several pharmaceutical

**SIMPLE AND SENSITIVE SPECTROPHOTOMETRIC ASSAY OF ISONIAZID IN PHARMACEUTICALS USING PERMANGANATE, METHYL ORANGE AND INDIGO CARMINE AS REAGENT**

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1Department of Chemistry, University of Mysore, Manasagangotri, Mysuru-570 006, Karnataka, India
2Department of Chemistry, UBDT College of Engineering, Davanagere-577 004, Karnataka, India

**Abstract:** Two simple, sensitive and rapid spectrophotometric methods are described for the determination of isoniazid (INH) in pharmaceuticals. The methods are based on the oxidation of INH by a measured excess of KMnO₄ in acid medium followed by the determination of residual oxidant by reacting with either methyl orange and measuring the absorbance at 520 nm (method A) or indigo carmine, when the absorbance was measured at 610 nm (method B). Experimental conditions such as KMnO₄ and dye concentrations, acid medium and reaction time to yield better performance characteristics were carefully studied and optimized. Beer’s law was obeyed in the concentration ranges, 0.25-5.0 and 0.25-4.0 µg/mL for method A and method B, respectively, and the corresponding molar absorptivity values were 2.5 × 10⁵ and 3.0 × 10⁵ L/M cm⁻¹. The limits of detection (LOD) and quantification (LOQ) were 0.08 and 0.23 µg/mL (method A) and 0.16 and 0.18 µg/mL (method B). Intra-day and inter-day accuracy expressed as relative error were better than 3% and the respective precision reported as relative standard deviation were also < 3%. Methods, when applied to tablets, yielded results which compared well with the label claim and also with those obtained by the reference method. No interference was observed from the tablet excipients.

**Keywords:** isoniazid, assay, spectrophotometry, KMnO₄, dyes, pharmaceuticals

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**Isoniazid (INH)** (Fig. 1), chemically known as pyridine-4-carboxylic acid hydrazide, is an antitubercular drug now widely used together with rifampicin and streptomycin for the chemotherapy of tuberculosis. This has prompted many investigators to devise methods for its determination in its pure form as well as in its tablet form.

The drug is official in Indian Pharmacopoeia (IP) (1), British Pharmacopoeia (BP) (2) and United State Pharmacopeia (USP) (3). IP and BP describe titration of the drug with potassium bromate in the presence of potassium bromide using methyl red indicator. USP describes HPLC method using L1 column (4.6 mm × 25 cm) and a mobile phase consisting of methanol : water (40 : 60) (pH adjusted to 2.5 with H₂SO₄) with a flow rate of 1.5 mL/min and UV-detection at 254 nm. Apart from the above official methods, a number of methods based on several techniques are found in the literature for INH and include: titrimetry (4-7), voltammetry (8-13), ion selective electrode-potentiometry (14-19), amperometry (20, 21), spectrofluorimetry (22, 23), chemiluminescence spectrometry (24-33), high performance liquid chromatography (HPLC) (34-37), gas chromatography (GC) (38-40), LC/LC-MS (41) and capillary electrophoresis (42-44).

Visible spectrophotometry is by far the most widely used technique for the assay of INH. Methods based on a variety of reaction schemes such as, nucleophilic substitution (45), condensation (46-49), charge-transfer and ion-pair (50), derivatization (51-54), diazo-coupling (55, 56), oxidative coupling (57, 58), complex formation (59-61) and redox followed by complexation (62-65). These methods suffer from the disadvantages such as drastic experimental conditions, use of organic solvent, longer standing time, poor sensitivity, narrow linear range, etc.

KMnO₄ has been a very useful reagent for the spectrophotometric assay of several pharmaceutical
The present paper deals with the application of KMnO₄ and two dyes, methyl orange and indigo carmine for the rapid and sensitive assay of INH in pharmaceuticals. In this work, INH was treated with a known excess of KMnO₄ and the unreacted oxidant was determined by reacting with either methyl orange followed by the absorbance measured at 520 nm (method A) or indigo carmine, where the absorbance was measured at 610 nm (method B). The developed methods offer the advantages of simplicity, speed, and sensitivity without the need for expensive chemicals and instrumentation.

EXPERIMENTAL

Apparatus
Absorbance measurements were made using Systronics model 166 digital spectrophotometer (Systronics, Ahmedabad, Gujarat, India) equipped with 10-mm matched quartz cells.

Reagents and materials
All the chemicals used were of analytical reagent grade and distilled water was used throughout the investigation. Pure INH was procured from Cipla India Ltd., Bangalore, India and used as received. Isonex-100, Isonex forte-300 and Tubernex forte-300 tablets were purchased from local commercial sources.

Potassium permanganate: An approximately 0.01 M solution was prepared by dissolving about 0.4 g of KMnO₄ (Merck Ltd., Mumbai, India) in water, and diluted to 250 mL in a calibrated flask, and standardized using H.A Bright’s procedure (77). The standard solution was diluted appropriately with water to get 60 and 70 µg/mL for use in method A and method B, respectively.

Methyl orange (MO): A standard solution equivalent to 500 µg/mL methyl orange was prepared by dissolving 58.8 mg of dye (S. D. Fine Chem., Mumbai, India, 85% dye content) in water and diluted to 100 mL in a calibrated flask; and filtered using Whatman number 42 filter paper. It was diluted to get a 50 µg/mL dye solution for use in method A.

Indigo carmine (IC): A standard solution containing 1000 µg/mL indigo carmine was prepared by dissolving 107.6 mg of indigo carmine (Loba Chemie Pvt. Ltd., Mumbai, India, 93% dye content) in water and diluting to the mark in a 100 mL calibrated flask. The solution was diluted with water to get a working concentration of 200 µg/mL for use in method B.

Sulphuric acid (5 M): Concentrated acid (S.D. Fine Chem, Mumbai, India, sp. gr. 1.84) was appropriately diluted with water to get the required concentration.

Standard INH solution: Ten mg of INH was dissolved in water and made up to 100 mL in a calibrated flask, and then diluted to 10 µg/mL.

Assay procedures
Procedure for bulk drug
Method A (using methyl orange): Different aliquots (0.25, 0.5, 1.0, 5.0 mL) of 10 µg/mL INH solution were accurately transferred into a series of 10 mL calibrated flasks and the total volume was adjusted to 5.0 mL by adding adequate quantity of water. To each flask, 1.0 mL of 5 M H₂SO₄ was added followed by 1.0 mL of 60 µg/mL KMnO₄. The flasks were stoppered, content mixed and allowed to stand for 5 min with occasional shaking. Then, 1.0 mL of methyl orange solution (50 µg/mL) was added to each flask, and after 5 min, the mixture was diluted to the volume with water and mixed well. The absorbance of each solution was measured at 520 nm against the reagent blank.

Method B (using indigo carmine): Varying aliquots (0.25, 0.5, 1.0, 4.0 mL) of 10 µg/mL INH standard solution were transferred into a series of 10 mL calibrated flasks using a micro burette and the total volume was brought to 4 mL by adding adequate quantity of water. To each flask 1 mL of 5 M H₂SO₄ and 1.0 mL of KMnO₄ (70 µg/mL) were added. The content was mixed well and the flasks were kept aside for 5 min with intermittent shaking. Finally, 1.0 mL of indigo carmine solution (200 µg/mL) was added to each flask and the volume was adjusted to the mark with water. The absorbance of each solution was measured at 610 nm against a reagent blank.

A standard graph was prepared by plotting absorbance against concentration and the unknown concentration was computed from the regression equation derived using Beer’s law data.

Procedure for tablets
Ten tablets were weighed and pulverized. An amount of tablet powder containing 10 mg INH was transferred into a 100 mL volumetric flask. The con-
tent was shaken well with 60 mL of water and diluted to the mark with water. The insoluble residue was filtered off using Whatman No. 42 filter paper. First 10 mL portion of the filtrate was discarded and a subsequent portion was diluted to get a working concentration of 10 µg/mL and subjected to analysis following the general procedure described earlier.

**Procedure for placebo blank and synthetic mixture**

A placebo blank of the composition acacia (15 mg), hydroxyl cellulose (10 mg), magnesium stearate (15 mg), starch (10 mg), sodium citrate (10 mg), sodium alginate (10 mg) and talc (20 mg) was prepared by thorough mixing. Ten mg of the placebo was taken and its solution prepared as described under “Procedure for tablets” and then analyzed using the procedures described above. To 10 mg of the placebo blank, 5 mg of INH was added and homogenized, transferred to 50 mL volumetric flask and the solution was prepared as described under “Procedure for tablets”. Synthetic mixture solution (100 µg/mL in INH) was diluted to 10 µg/mL level and 2 mL aliquots were taken in five replicates and assayed following the general procedures.

**RESULTS AND DISCUSSION**

The present work involves the oxidation of INH by a measured excess of KMnO₄ in H₂SO₄ medium followed by determination of surplus KMnO₄ after the reaction is ensured to be complete. The unreacted KMnO₄ is determined by reacting with a fixed amount of either MO and measuring the absorbance at 520 nm or IC and measuring the absorbance at 610 nm. In both the methods, the amount of KMnO₄ reacted is related to the amount of INH. The methods make use of the bleaching action of KMnO₄ on the dyes, where the discoloration is caused by oxidative destruction of the dye. Possible reaction pathway is shown in Scheme 1.

Many dyes are irreversibly destroyed to colourless species by oxidizing agents in acid medium (78) and this observation has been exploited for the indirect spectrophotometric determination of some pharmaceutical compounds (79-85). In the proposed methods, the ability of KMnO₄ to effect the oxidation of INH and irreversibly destroy MO and IC to colourless products in acid medium has been capitalized. In either method, the absorbance increased linearly with increasing concentration of drug. INH when added in increasing concentrations to a fixed concentration of KMnO₄ consumes the latter and there will be a concomitant decrease in the concentration of KMnO₄. When a fixed concentration of either dye is added to decreasing concentrations of KMnO₄, a concomitant increase in the concentration of dye is obtained. This is observed as a proportional increase in the absorbance at the respective wavelengths of maximum absorption with increasing concentration of INH (Figs. 2, 3).

**Optimization of KMnO₄ and dye concentrations**

Preliminary experiments were performed to fix the upper limits of the methyl orange (MO) and indigo carmine (IC) that would produce a reasonably high absorbance, and these were found to be 5 µg/mL MO in method A and 20 µg/mL IC in method B. To fix the optimum concentration of KMnO₄, different concentrations of KMnO₄ were reacted with a fixed concentration of MO (5 µg/mL) or IC (20 µg/mL) in H₂SO₄ medium, and the absorbance was measured at 520 or 610 nm. A constant and minimum absorbance resulted with 6.0 and 7.0 µg/mL KMnO₄ for method A and method B, respectively. Different concentrations of INH were reacted with 1 mL of 60 µg/mL KMnO₄ in method A and 70 µg/mL KMnO₄ in method B. The amount of KMnO₄ reacted is related to the amount of INH. The methods make use of the bleaching action of KMnO₄ on the dyes, where the discoloration is caused by oxidative destruction of the dye. Possible reaction pathway is shown in Scheme 1.
in method B in H$_2$SO$_4$ medium before determining the residual KMnO$_4$. This facilitated the optimization of the linear dynamic range over which each method could be applied for the assay of INH.

**Effect of reaction medium**

The reaction between INH and KMnO$_4$ was performed in different acid media viz. sulphuric acid, hydrochloric acid and perchloric acid. Sulphuric acid was found to be the ideal medium for the oxidation of INH by KMnO$_4$ as well as the latter’s determination employing either dye. The effect of acid concentration on the reaction between INH and KMnO$_4$ was studied by varying the concentration of H$_2$SO$_4$ keeping the concentrations of KMnO$_4$ and drug fixed. Higher acid concentrations showed lower sensitivity, hence, 1 mL of 5 M H$_2$SO$_4$ in a total volume of 10 mL was fixed as optimum.

**Study of reaction time and stability**

Under the described experimental conditions, for a quantitative reaction between INH and KMnO$_4$, contact time of 5 min was found necessary in both methods at room temperature. After addition of dye, the reaction between KMnO$_4$ and dye was instantaneous and absorbance of the unreacted dye was stable at least for 45 min in method A and 60 min in method B.

**Method validation**

**Linearity and sensitivity**

The proposed methods were validated for linearity, selectivity, precision, accuracy, robustness.

![Figure 1. Structure of INH](image)

![Figure 2. Absorption spectra of 6 µg/mL KMnO$_4$ after reacting with: a. 2; b. 3; c. 4 and d. 5 µg/mL INH](image)
and ruggedness, and recovery. In both the methods, a linear correlation (Fig. 4) was found between absorbance at $\lambda_{\text{max}}$ and concentration of INH in the ranges given in Table 1. Regression analysis of the Beer’s law data using the method of least squares was made to evaluate the slope (b), intercept (a) and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$, nm</td>
<td>520</td>
<td>610</td>
</tr>
<tr>
<td>Beer’s law limits, µg/mL</td>
<td>0.25 – 5.0</td>
<td>0.25 – 4.0</td>
</tr>
<tr>
<td>Molar absorptivity, L/M cm$^1$</td>
<td>$2.5 \times 10^4$</td>
<td>$3.0 \times 10^4$</td>
</tr>
<tr>
<td>Sandell sensitivity*, µg cm$^2$</td>
<td>0.0055</td>
<td>0.0045</td>
</tr>
<tr>
<td>Limit of detection, mg/mL</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>Limit of quantification, mg/mL</td>
<td>0.23</td>
<td>0.18</td>
</tr>
<tr>
<td>Regression equation, $Y**$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept, (a)</td>
<td>0.0192</td>
<td>0.0102</td>
</tr>
<tr>
<td>Slope, (b)</td>
<td>0.1666</td>
<td>0.2167</td>
</tr>
<tr>
<td>Standard deviation of intercept ($S_a$)</td>
<td>0.0045</td>
<td>0.0021</td>
</tr>
<tr>
<td>Standard deviation of slope ($S_b$)</td>
<td>0.0109</td>
<td>0.0226</td>
</tr>
<tr>
<td>Regression coefficient (r)</td>
<td>0.9989</td>
<td>0.9990</td>
</tr>
</tbody>
</table>

*Limit of determination as the weight in µg/mL of solution, which corresponds to an absorbance of $A = 0.001$ measured in a cuvette of cross-sectional area 1 cm$^2$ and $l = 1$ cm. $Y = a + bX$, where $Y$ is the absorbance, $X$ concentration in µg/mL.
correlation coefficient \( (r) \) for each system and the values obtained from this investigations are also presented in Table 1. Sensitivity parameters such as apparent molar absorptivity and Sandell sensitivity and the limits of detection and quantification are calculated as per the current ICH guidelines and are

![Figure 4. Calibration curves for: a) method A; b) method B](image)

Table 2. Results of intra-day and inter-day accuracy and precision study.

<table>
<thead>
<tr>
<th>Method</th>
<th>INH taken</th>
<th>Intra-day accuracy and precision ((n = 7))</th>
<th>Inter-day accuracy and precision ((n = 5))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INH found*</td>
<td>%RE</td>
<td>%RSD</td>
</tr>
<tr>
<td>A</td>
<td>1.0</td>
<td>1.02</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.96</td>
<td>2.27</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.04</td>
<td>1.31</td>
</tr>
<tr>
<td>B</td>
<td>2.0</td>
<td>1.98</td>
<td>1.01</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.04</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.05</td>
<td>1.27</td>
</tr>
</tbody>
</table>

RE - Relative error and RSD - Relative standard deviation; INH taken and found are in µg/mL; Mean value of \( n \) determinations.

Table 3. Results of robustness and ruggedness expressed as intermediate precision (\% RSD).

<table>
<thead>
<tr>
<th>Method</th>
<th>Robustness</th>
<th>Ruggedness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal concentration ((n=3))</td>
<td>Reaction times* ((n=3))</td>
</tr>
<tr>
<td>A</td>
<td>1.0</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>3.38</td>
</tr>
<tr>
<td>B</td>
<td>2.0</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>2.08</td>
</tr>
</tbody>
</table>

* Reaction time was 4, 5 and 6 min.; * Volume of H\(_2\)SO\(_4\), 0.9, 1.0 and 1.1 mL.
compiled in Table 1 and speak of the excellent sensitivity of the proposed methods.

**Accuracy and precision**

In order to assess the precision and accuracy of the proposed methods, pure INH at three concentration levels within the linearity range were analyzed, each determination being repeated seven times (intra-day precision) on the same day and one time each for five successive days (inter-day precision). The percent relative standard deviation (% RSD) was $1.34\%$ (intra-day) and $2.10\%$ (inter-day). In addition, the accuracy of the proposed method was evaluated by calculating the percentage relative error (% RE), which varied between $1.01\%$ and $3\%$. The results of this study compiled in Table 2 indicate the high accuracy and precision of the proposed methods.

**Selectivity**

Selectivity was evaluated by both placebo blank and synthetic mixture analyses. The placebo blank, whose composition was given earlier was prepared and analyzed as described under the recommended procedures. The resulting absorbance readings for the methods were the same as the reagent blanks, inferring no interference from the placebo. The selectivity of the methods was further confirmed by carrying out recovery study from synthetic mixture. The percent recoveries of INH were $98.7 \pm 1.18$ and $101.4 \pm 1.63$ for method A and method B, respectively. This confirms the selectivity of the proposed methods in the presence of the commonly employed tablet excipients.

**Robustness and ruggedness**

To evaluate the robustness of the methods, two important experimental variables, viz. the amount of acid and reaction time, were slightly varied, and the capacity of the methods was found to remain unaffected by small deliberate variations. The results of this study are presented in Table 3 and indicate that the proposed methods are robust. Method ruggedness is expressed as %RSD of the same procedure

---

**Table 4. Results of analysis of tablets by the proposed methods and statistical comparison with the official method.**

<table>
<thead>
<tr>
<th>Tablet studied</th>
<th>Label claim* mg/tablet</th>
<th>Founda (Percent of label claim ± SD)</th>
<th>Reference method</th>
<th>Proposed methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Method A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Method B</td>
</tr>
<tr>
<td>Isonex1</td>
<td>100</td>
<td>99.36 ± 1.65</td>
<td></td>
<td>98.84 ± 1.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t = 2.72$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F = 1.35$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>98.75 ± 1.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t = 2.82$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F = 1.42$</td>
</tr>
<tr>
<td>Isonex forte1</td>
<td>300</td>
<td>97.99 ± 1.03</td>
<td></td>
<td>99.07 ± 1.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t = 1.51$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F = 1.4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>98.63 ± 1.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t = 0.95$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F = 1.12$</td>
</tr>
<tr>
<td>Tubernex forte2</td>
<td>300</td>
<td>100.5 ± 0.81</td>
<td></td>
<td>101.2 ± 1.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t = 1.02$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F = 2.54$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100.9 ± 1.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t = 0.64$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$F = 2.02$</td>
</tr>
</tbody>
</table>

* Mean value of five determinations; the value of $t$ and $F$ (tabulated) at 95% confidence level and for four degrees of freedom are $2.77$ and $6.39$, respectively. *Marketed by: $1$ Pfizer Limited (Pharmacia India Pvt. Ltd.); $2$ Radicura Pharmaceuticals Pvt. Ltd. India.

**Table 5. Results of recovery study via standard addition method.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Tablet studied</th>
<th>INH in tablet µg/mL</th>
<th>Pure INH added µg/mL</th>
<th>Total found µg/mL</th>
<th>Pure INH recovered* Percent ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A</td>
<td>Isonex-100</td>
<td>1.48</td>
<td>0.75</td>
<td>2.18</td>
<td>97.85 ± 1.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.48</td>
<td>1.50</td>
<td>3.02</td>
<td>101.3 ± 0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.48</td>
<td>2.25</td>
<td>3.82</td>
<td>102.5 ± 1.76</td>
</tr>
<tr>
<td>Method B</td>
<td>Isonex-100</td>
<td>1.48</td>
<td>0.75</td>
<td>2.19</td>
<td>98.55 ± 0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.48</td>
<td>1.50</td>
<td>2.95</td>
<td>99.26 ± 0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.48</td>
<td>2.25</td>
<td>2.75</td>
<td>100.9 ± 0.84</td>
</tr>
</tbody>
</table>

*Mean value of three determinations.
Table 6. Comparison of performance characteristics of the proposed methods with the existing spectrophotometric methods.

<table>
<thead>
<tr>
<th>No.</th>
<th>Reagent's used</th>
<th>Methodology</th>
<th>( \lambda_{	ext{max}} ) (nm)</th>
<th>Linear range (µg/mL) ((e = L/M/cm))</th>
<th>Remarks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Epichlorohydrine</td>
<td>Measurement of purple colored coupled complex</td>
<td>405</td>
<td>2 – 22 ((0.51 \times 10^4))</td>
<td>Longer reaction time and heating</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Hydroxyphenacylchloride</td>
<td></td>
<td>402</td>
<td>20 – 120 ((0.10 \times 10^4))</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>Ethyl vanillin</td>
<td>Measurement of yellow colored hydrazone complex</td>
<td>410</td>
<td>2 – 16 ((7.1 \times 10^3))</td>
<td>-</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>Vanillin</td>
<td>Measurement of yellow colored hydrazone complex</td>
<td>405</td>
<td>1 – 12</td>
<td>-</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>Isatin</td>
<td>Measurement of yellow colored hydrazone complex</td>
<td>340 ((1.2 \times 10^5))</td>
<td>0 – 32</td>
<td>Requires close pH control and 40 min. standing time, measurement at a shorter wavelength</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>Sodium 1,2-naphthoquinone-4-sulfonate &amp; cetyltrimethyl ammonium bromide</td>
<td>Absorbance of condensation product measured</td>
<td>500</td>
<td>2.0 – 5.6</td>
<td>Employ a costly reagent</td>
<td>49</td>
</tr>
<tr>
<td>6</td>
<td>Chlaronic acid Tetracyanoethylene 2,3-dichloro-5,6-dicyano-1,4-benzoquinone</td>
<td>Measurement of charge-transfer complex</td>
<td>500</td>
<td>1.37 – 8.2</td>
<td>Use of organic solvent, requires heating step</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Thymol blue Bromophenol blue Bromocresol green</td>
<td>Measurement of ion-associate complex</td>
<td>480</td>
<td>6.85 – 34.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>390</td>
<td>10.96 – 21.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>410</td>
<td>1.37 – 6.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>320</td>
<td>1.37 – 8.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2-Hydroxy-1,4-naphthoquinone</td>
<td>Measurement of derivatized product</td>
<td>365</td>
<td>5 – 25</td>
<td>Requires close pH control, use of non-aqueous medium, measurement at a shorter wavelength</td>
<td>51</td>
</tr>
<tr>
<td>8</td>
<td>1, 2-Naphthoquinone-4-sulfonate</td>
<td>Measurement of pink colored condensed product</td>
<td>495</td>
<td>0.5 – 3.0 ((1.18 \times 10^4))</td>
<td>Requires close pH control</td>
<td>52</td>
</tr>
<tr>
<td>9</td>
<td>6-Methyl1-2-pyridinecarboxaldehyde</td>
<td>Measurement of hydrazine derivative</td>
<td>328</td>
<td>2 – 16</td>
<td>Measurement at lower analytical wavelength</td>
<td>53</td>
</tr>
<tr>
<td>No.</td>
<td>Reagent/s used</td>
<td>Methodology</td>
<td>( \lambda_{	ext{max}} ) (nm)</td>
<td>Linear range (µg/mL) ((ε = L/M/cm))</td>
<td>Remarks</td>
<td>References</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------------------------</td>
<td>---------------------------------------------</td>
<td>---------------------------------</td>
<td>------------------------------------------</td>
<td>---------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>10</td>
<td>9-Chloroacridine</td>
<td>Measurement of absorbance of derivatized complex</td>
<td>500</td>
<td>-</td>
<td>Requires heating step, time consuming</td>
<td>54</td>
</tr>
<tr>
<td>11</td>
<td>4,4’-Methylene-bis-m-nitroaniline</td>
<td>Measurement of purple colored diazo-coupled complex</td>
<td>495</td>
<td>0.1 – 15 (5.63 \times 10^6)</td>
<td>Requires low temperature</td>
<td>55</td>
</tr>
<tr>
<td>12</td>
<td>4,4’-Sulfonyldianiline</td>
<td>Measurement of purple colored diazo-coupled complex</td>
<td>440, 335</td>
<td>0.5 – 20 (5.72 \times 10^6)</td>
<td>Requires low temperature</td>
<td>56</td>
</tr>
<tr>
<td>13</td>
<td>Tiron-NaIO₄</td>
<td>Measurement of red colored oxidative coupled product</td>
<td>507</td>
<td>1.0 – 15 (1.84 \times 10^6)</td>
<td>Use of multi step reaction system</td>
<td>57</td>
</tr>
<tr>
<td>14</td>
<td>Tiron-KIO₄</td>
<td>Measurement of red colored oxidative coupled product</td>
<td>505</td>
<td>1.5 – 18 (1.77 \times 10^6)</td>
<td>Use of multi step reaction system</td>
<td>58</td>
</tr>
<tr>
<td>15</td>
<td>Uranyl acetate</td>
<td>Measurement of yellow colored uranyl isonicotinylidithiocarbazate complex</td>
<td>410</td>
<td>-</td>
<td>Multi step reaction, time consuming, use of expensive chemical</td>
<td>59</td>
</tr>
<tr>
<td>16</td>
<td>Cu(II) &amp; neocuproine</td>
<td>Measurement of complex</td>
<td>454</td>
<td>0.3 – 3.5</td>
<td>Requires close pH control, a multi step reaction</td>
<td>60</td>
</tr>
<tr>
<td>17</td>
<td>Rose bengal</td>
<td>Measurement of acetone-chloroform extractable ion-pair complex</td>
<td>555</td>
<td>2.8 – 5.6</td>
<td>Requires time consuming and tedious extraction step, use of organic solvent</td>
<td>61</td>
</tr>
<tr>
<td>18</td>
<td>*NBS-iodide-starch</td>
<td>Absorbance of starch-iodine complex measured</td>
<td>572</td>
<td>0.1 – 3.4</td>
<td>Multi step reaction, employs an unstable oxidant</td>
<td>62 &amp; 63</td>
</tr>
<tr>
<td>19</td>
<td>Iron(III)-ferricyanide</td>
<td>Measurement of Prussian blue</td>
<td>735</td>
<td>0.04 – 8 (3.92 \times 10^6)</td>
<td>-</td>
<td>64</td>
</tr>
<tr>
<td>20</td>
<td>Iron(III)-PDT*</td>
<td>Measurement of complex</td>
<td>558</td>
<td>0.62 – 6.15</td>
<td>Multi step reaction, requires an expensive reagent</td>
<td>65</td>
</tr>
<tr>
<td>21</td>
<td><strong>a) KMnO₄-MO</strong></td>
<td>Unbleached color of the dye was measured</td>
<td>520</td>
<td>0.25 – 5.0 (2.5 \times 10^6)</td>
<td>Simple, highly sensitive, no heating &amp; no extraction step</td>
<td>Present work</td>
</tr>
<tr>
<td></td>
<td><strong>b) KMnO₄-JC</strong></td>
<td></td>
<td>610</td>
<td>0.25 – 4.0 (3.0 \times 10^6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\*NBS - N-bromosuccinimide, KMnO₄ - potassium permanganate, FC reagent - Folin-Ciocalteau reagent, PDT - 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine.
applied by three analysts using the same instrument and also by a single analyst using three different spectrophotometers. The inter-analysts’ and inter-instruments’ RSD values were = 3.04% indicating ruggedness of the proposed methods. The results of this study are presented in Table 3.

Application to tablets
The results presented in Table 4 showed that there was a close agreement between the results obtained by the proposed methods and the label claim. The results were also compared with those of the reference method (2) statistically by Student’s t-test for accuracy and variance ratio F-test for precision at 95% confidence level. The calculated t- and F-values indicated that there was no significant difference between the proposed methods and the reference method with respect to accuracy and precision.

Accuracy by recovery study
To further ascertain the accuracy of the proposed methods, a standard addition procedure was followed. A fixed amount of pre-analyzed tablet powder was taken and pure drug at three different levels (50, 100 and 150% of that in tablet powder) was added. The total was determined by the proposed methods. The determination at each level was repeated three times and the percent recovery of the added standard was calculated. Results of this study are presented in Table 5 and are reflective of the high accuracy, selectivity and reliability of the proposed methods for routine use.

CONCLUSIONS
Two rapid, simple, and selective spectrophotometric methods for the determination of isoniazid in pharmaceuticals are described. The methods are free from rigid experimental conditions such as critical pH control, heating, extraction, etc. They employ inexpensive and easily available chemicals and instrument compared to most methods reported previously (Table 6), making them cost-effective. The methods are applicable over wide linear dynamic ranges and highly sensitive (ɛ = 104) compared to the existing spectrophotometric methods. The methods can be used as alternatives to highly sophisticated instrumental methods reported for isoniazid.

Acknowledgments
Authors express their gratitude to the quality control manager, Cipla Ltd., Bangalore, India, for gift sample of pure isoniazid and the authorities of the University of Mysore, Mysore, for permission and facilities. Prof. K. Basavaiah thanks University Grants Commission New Delhi, India, for the award of UGC-BSR faculty fellowship.

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3. The United States Pharmacopoeia, XXIV Revision, the National Formulary XIX, USP Convention, Rockville 2000.

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Quantitative structure–retention relationships (QSRRs) describe relationships between molecular descriptors and chromatographic properties of molecules. The QSRR approach can be applied: (1) for prediction of retention; (2) for identification of the most revealing structural descriptors (regarding properties); (3) to gain insight into the retention mechanism of separation; (4) for the evaluation of complex physicochemical properties (other than chromatographic) of analytes, e.g., lipophilicity; and (5) for the prediction of relative biological activity (1-4).

Micellar liquid chromatography (MLC) is a reversed-phase liquid chromatographic (RPLC) mode with a mobile phase consisting of an aqueous solution of surfactant above its critical micellar concentration (CMC). Chromatographic properties of nine macrolide antibiotics have been studied on cyano-bonded silica stationary phase. Four surfactants, with different chemical character: anionic (SDS) cationic (CTAB and SC) and nonionic (triton X-100), were tested as modifiers of mobile phase. The influence of physicochemical parameters of tested macrolide antibiotics on the retention were investigated utilizing quantitative structure–retention relationships (QSRR) approach. Molecular descriptors were calculated from the optimized structures using HyperChem and ChemAxon software. Additionally, the quantitative structure–activity relationships (QSAR) equations, based on chromatographic parameters, were proposed. The QSAR models can be used for predictions of antimicrobial activity of *Staphylococcus aureus, Streptococcus pneumoniae* and *Listeria monocytogenes* against.

**Keywords:** micellar liquid chromatography (MLC), Quantitative Structure-Retention Relationships (QSRR), Quantitative Structure-Activity Relationships (QSAR), macrolide antibiotics
Additionally, the utilization of obtained chromatographic parameters in QSAR analysis were examined.

EXPERIMENTAL

Analytes
The 9 reference standards (dirithromycin, josamycin, erythromycin, azithromycin, clarithromycin, roxithromycin, spiramycin, troleandomycin and tylosin) used during the study were purchased from Sigma-Aldrich (Steinheim, Germany).

Mobile and stationary phases
Chromatographic analyses were performed on silica gel 60 CN F 254s HPTLC plates manufactured by Merck (Darmstadt, Germany). The organic solvents (HPLC-grade: acetone, acetonitrile, dioxane and methanol) were supplied by Sigma-Aldrich (Gillingham, Dorset, UK). Water was purified using Millipore Direct-Q 3 UV Water Purification System (Millipore Corporation, Bedford, MA, USA). The surfactants: sodium dodecyl sulfate (SDS), cetrimonium bromide (CTAB), Triton X-100 and sodium cholate (SC) were purchased from Sigma-Aldrich (Steinheim, Germany). The mobile phases were prepared by mixing appropriate quantities of pure organic solvents and aqueous solution of surfactant in proportion listed bellow:

a) 50, 65, 80, 95, 110 mM aqueous solution of SDS with 15 % \((v/v)\) addition of acetonitrile
b) 85, 95, 110, 125 mM aqueous solution of CTAB with 15 % \((v/v)\) addition of acetonitrile
c) 30, 40, 50, 60, 70 mM aqueous solution of SC with 15 % \((v/v)\) addition of acetonitrile

Chromatographic analysis
Macrolide antibiotics were dissolved in methanol at a concentration of 5 mg/mL and 1 µl of the obtained solutions were spotted with the used of micropipettes on the plates. 65 mm wide and 105 mm high cylindrical glass chambers (Sigma-Aldrich; Steinheim, Germany) were used to develop the plates. The chromatographic chamber was saturated 20 minutes before every analysis. Chromatograms were developed by the ascending technique, at room temperature \((20 ± 2°C)\) to the distance of 8 cm. The studied substances were visualized by spraying the plates with the mixture of concentrated sulfuric acid and methanol \((1 : 4, v/v)\). After spraying, the plates were heated at 121°C for 10 min (Camag TLC Plate Heater III). Subsequently, the eluted compounds were visible in UV light \((254 \text{ and } 366 \text{ nm wavelength})\) as colored spots. The chromatographic analyses were performed in triplicate and mean retardation factor \((R_f)\) values were calculated.

Chromatographic parameters
The \(R_f\) parameters were calculated with use of Bate-Smith and Westall formula:

\[
R_m = \log \left(\frac{1}{R_f} - 1\right) \quad (1)
\]

The correlation between chromatographic parameter \(k\) and surfactant concentration in the mobile phase is presented with Foley formula (10):

\[
\frac{1}{k} = \frac{1}{k_m} + \frac{K_{AM}}{k_m} [M] \quad (2)
\]

where \([M]\) is the total concentration of surfactant in the mobile phase, \(K_{AM}\) is the constant describing solute–micelle binding and \(k_m\) is the solute retention parameter at zero micellar concentration, i.e. at surfactant monomer concentration equal to the CMC \((6)\). The Foley formula can be applied for aqueous solutions of surfactant or mobile phases with the equal. The parameters \(K_{AM}\) and \(k_m\) can be calculated from the slope and intercept of experimental \(1/k\) versus \([M]\) relationships \((6, 10, 11)\).

Table 1. The physicochemical parameters of the tested macrolide antibiotics.

<table>
<thead>
<tr>
<th></th>
<th>(\log D)</th>
<th>(\log D)</th>
<th>(H_m)</th>
<th>(E_{DREID})</th>
<th>(\sigma_{min})</th>
<th>(\mu)</th>
<th>Energy_{Dreiding}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>-0.74</td>
<td>1.40</td>
<td>14</td>
<td>-42.00</td>
<td>-0.35</td>
<td>5.57</td>
<td>218.34</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.71</td>
<td>2.41</td>
<td>14</td>
<td>-41.89</td>
<td>-0.31</td>
<td>12.69</td>
<td>223.45</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>-0.79</td>
<td>1.99</td>
<td>16</td>
<td>-42.02</td>
<td>-0.35</td>
<td>6.05</td>
<td>277.79</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.38</td>
<td>2.08</td>
<td>14</td>
<td>-41.50</td>
<td>-0.35</td>
<td>10.64</td>
<td>242.31</td>
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<tr>
<td>Josamycin</td>
<td>2.09</td>
<td>3.06</td>
<td>16</td>
<td>-42.37</td>
<td>-0.37</td>
<td>7.41</td>
<td>189.70</td>
</tr>
<tr>
<td>Roxythromycin</td>
<td>1.28</td>
<td>2.98</td>
<td>17</td>
<td>-41.87</td>
<td>-0.35</td>
<td>10.06</td>
<td>224.17</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>-0.92</td>
<td>1.58</td>
<td>16</td>
<td>-42.03</td>
<td>-0.38</td>
<td>3.01</td>
<td>245.26</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>1.09</td>
<td>2.79</td>
<td>16</td>
<td>-42.69</td>
<td>-0.32</td>
<td>9.82</td>
<td>324.64</td>
</tr>
<tr>
<td>Tylosin</td>
<td>1.49</td>
<td>3.02</td>
<td>18</td>
<td>-42.21</td>
<td>-0.33</td>
<td>7.95</td>
<td>276.56</td>
</tr>
</tbody>
</table>
Molecular descriptors

The structures of tested compounds were constructed using the Open Babel 2.3.3 software (12), HyperChem 8.08 software with ChemPlus Extension (Hypercube, Waterloo, Canada) was used for the calculation of molecular bulkiness-related descriptors and molecular polarity-related (electronic) descriptors. In the first step, the structures were optimized using the molecular mechanic calculations (MM+). In the next step, semi-empirical calculation method Austin Model 1 (AM1) was applied. The number of hydrogen donors and acceptors were taken from PubChem database (13). Geometrical and topological descriptors were obtained with the use of ChemAxon software (14). All descriptors used in this study are presented in Table 1.

Determination of critical micelle concentrations

Critical micelle concentrations of CTAB and SC were determined by the surface tension measurements on an Easy Dyne (Krüss, Germany) tensiometer. Solutions of known concentration were progressively diluted and examined with the use of Wilhelmy plate method. Temperature was kept at 25 ± 0.2°C using a thermostated circulating water bath. Before each measurement, the Wilhelmy plate was washed with pure water and then heated to redness using a Bunsen burner. All samples were prepared in 15% solution of ACN in water. The CMCs values were calculated by plotting the surface tension against the log of the concentration of surfactants. The accuracy of the measurement was within ± 0.1 mN/m.

Data analysis

All statistical calculations were performed using STATISTICA 9.1 (StatSoft, Tulsa, Oklahoma, USA). Correlations between molecular descriptors and retention or biological activities were presented as QSRR/QSAR equations. In order to establish the QSRR/QSAR equations, the multiple linear regression (MLR) was derived, using stepwise regression. During calculations, the retention data were used as a dependent variables and structural parameters as the independent ones. Coefficients of correlation (r) and determination (R²), value of F-test and standard estimation error were used as the basis for testing the linearity of regression plots. All presented QSRR/QSAR equations were performed at a significant level of ≤ 5 %.

RESULTS AND DISCUSSIONS

In our research, a group of 9 selected macrolide antibiotics were used. All studied compounds, except for tylosin, belong to the same macrolide class in the Anatomical Therapeutic Chemical (ATC) classification system (J01F Macrolides, lincosamides and streptogramins). Tylosin is structurally similar to each other and it is used as a veterinary drug. The size of the tested group was limited but numbers of widely available analytical standards of macrolide derivatives were not much larger.

Chromatographic analysis

CN plates were chosen as stationary phase, because analysis on those plates is noticeably shorter in comparison to analysis on C18 or C8 bonded silica gel (6). Four surfactants were explored during this study. The surfactants have different chemical character and belong to various class: anionic (SDS) cationic (CTAB and SC) and nonionic (triton X-100). Preliminary chromatographic study showed that the addition of small amount of organic solvent to the aqueous solution of surfactant is necessary. When only aqueous solution of surfactants was used, the solutes remained at the starting point. This might be the consequence of high molecular mass and size of tested compounds. Four organic solvents, at different concentrations were tested: acetic acid, acetonitrile, dioxane and methanol.

Unfortunately, when triton X-100 was used as a modifier of mobile phase, the solutes remained on the starting line, although various proportion of triton X-100 and different organic modifiers were examined. Due to this fact, this chromatographic system was excluded from further analysis.

For further analyses of other chromatographic systems, the addition of 15 % (v/v) of acetonitrile in the mobile phase was selected, since it causes significant increase in Rf values. Additionally, acetonitrile significantly reduced the time of the analysis. On the other hand, the short-chain alcohol, as well as acetonitrile, in the mobile phase can lead to disintegration of micelles. For this reason, the concentration of surfactants was considerably higher than CMC in pure water. In the literature there is information that SDS micelles are stable in 20% solution of ACN (15) but there are no information about influences of ACN on CMC in case of using SC and CTAB. Therefore, the self-assembly abilities of CTAB and SC in 15% ACN have been investigated. Interesting might be the fact that ACN have only slight influence on CMC value in case of SC. The obtained CMC value is 11.5 mM, showing small increase in comparison to pure water (CMC value range between 2 – 6 mM). On the contrary, when CTAB was used, the CMC value increased extreme-
ly to the value of 83.6 mM in comparison to pure water (0.92 mM).

The obtained chromatographic parameters calculated according to equation 2 are listed in Table 2. Generally, the correlation coefficient was close to 0.95 confirming the applicability of the presented equation. Significantly high correlation was observed when SDS was used. When mobile phase included CTAB, the correlation coefficient was lower than 0.9 for josamycin ($r = 0.866$) and roxithromycin ($r = 0.878$). Moreover, clarithromycin has near linear behavior ($r = 0.891$) when mobile phase contained SC. The obtained results may suggest that for the cationic and the anionic surfactants the equation 2 represents the chromatographic behavior very well. The additional studies, based on a larger number of different surfactants should be performed to confirm the hypothesis.

Since parameters $K_{AM}/k_m$ and $1/k_m$ are analogical for $R_M$ and $m$ in classical reserved phase thin layer chromatography (RP-TLC), the inter-correlations were examined. The results of the analysis are showed in Table 3 and Figure 1. Generally, linear correlations of good statistical quality were observed, what is confirmed by high value of $r$, values of $F$-Snedecor’s test and small standard estimation error. This proves that, from the chromatographic point of view, the macrolide antibiotics can be regarded as one group of structurally similar analytes, and also suggests, that the mechanisms of chromatographic retention for the analyzed compounds are similar within their groups (9).

### Table 2. Parameters of Eq. (2) calculated for tested macrolide antibiotics with statistical parameters.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$K_{AM}/k_m$</th>
<th>$\sigma$</th>
<th>$k_m$</th>
<th>$\sigma$</th>
<th>$1/k_m$</th>
<th>$\sigma$</th>
<th>$r$</th>
<th>$R^2$</th>
<th>$F$</th>
<th>$s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>-0.16</td>
<td>0.085</td>
<td>0.52</td>
<td>0.114</td>
<td>0.956</td>
<td>0.914</td>
<td>21.27</td>
<td>0.038</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>-0.04</td>
<td>0.025</td>
<td>0.25</td>
<td>0.033</td>
<td>0.983</td>
<td>0.966</td>
<td>56.63</td>
<td>0.011</td>
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<td>0.018</td>
<td>0.42</td>
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<td>0.997</td>
<td>0.993</td>
<td>300.38</td>
<td>0.008</td>
<td></td>
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<tr>
<td>Erythromycin</td>
<td>-0.07</td>
<td>0.038</td>
<td>0.35</td>
<td>0.052</td>
<td>0.979</td>
<td>0.959</td>
<td>46.43</td>
<td>0.017</td>
<td></td>
<td></td>
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<td>Josamycin</td>
<td>0.00</td>
<td>0.014</td>
<td>0.06</td>
<td>0.018</td>
<td>0.928</td>
<td>0.861</td>
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<td>0.991</td>
<td>0.983</td>
<td>115.91</td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
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<td>0.53</td>
<td>0.037</td>
<td>0.995</td>
<td>0.991</td>
<td>211.02</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.017</td>
<td>0.22</td>
<td>0.023</td>
<td>0.989</td>
<td>0.979</td>
<td>93.102</td>
<td>0.008</td>
<td></td>
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<td>Tylosin</td>
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<td>0.029</td>
<td>0.30</td>
<td>0.039</td>
<td>0.983</td>
<td>0.966</td>
<td>57.173</td>
<td>0.013</td>
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<tr>
<td>Azithromycin</td>
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<td>0.047</td>
<td>0.26</td>
<td>0.064</td>
<td>0.947</td>
<td>0.897</td>
<td>17.81</td>
<td>0.020</td>
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<tr>
<td>Clarithromycin</td>
<td>0.16</td>
<td>0.050</td>
<td>0.40</td>
<td>0.067</td>
<td>0.975</td>
<td>0.951</td>
<td>32.38</td>
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<td>0.040</td>
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<td>0.966</td>
<td>54.29</td>
<td>0.013</td>
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<td>0.954</td>
<td>0.910</td>
<td>21.48</td>
<td>0.029</td>
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<tr>
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<td>0.019</td>
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<td>0.025</td>
<td>0.866</td>
<td>0.750</td>
<td>9.78</td>
<td>0.008</td>
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<td>0.771</td>
<td>6.22</td>
<td>0.019</td>
<td></td>
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<td>Spiramycin</td>
<td>0.45</td>
<td>0.032</td>
<td>0.04</td>
<td>0.004</td>
<td>0.930</td>
<td>0.865</td>
<td>12.82</td>
<td>0.019</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>0.43</td>
<td>0.016</td>
<td>-0.03</td>
<td>0.022</td>
<td>0.967</td>
<td>0.935</td>
<td>41.571</td>
<td>0.007</td>
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<td>Tylosin</td>
<td>0.30</td>
<td>0.043</td>
<td>0.21</td>
<td>0.059</td>
<td>0.909</td>
<td>0.826</td>
<td>10.385</td>
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<td>0.153</td>
<td>0.978</td>
<td>0.957</td>
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<td>0.891</td>
<td>0.794</td>
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<td>0.007</td>
<td>0.999</td>
<td>0.999</td>
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<td>0.879</td>
<td>21.76</td>
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<td>0.967</td>
<td>0.936</td>
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<td>0.071</td>
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<td>0.976</td>
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<td>0.059</td>
<td>-0.70</td>
<td>0.105</td>
<td>0.978</td>
<td>0.957</td>
<td>44.266</td>
<td>0.024</td>
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</tr>
<tr>
<td>Tylosin</td>
<td>0.91</td>
<td>0.062</td>
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<td>0.118</td>
<td>0.942</td>
<td>0.887</td>
<td>39.437</td>
<td>0.038</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$r$ - the coefficient of correlation; $R^2$ - the coefficient of determination; $F$ - the value of Snedecor F-test; $s$ - the standard estimation error; $p$-value < 0.05.
QSRR analysis

In QSRR study, the predictor variable is the retention constant, in the case of TLC – it is $K_{\text{AM}}$ parameter, and the independent variables are molecular descriptors. In order to establish QSRR models MLR was applied. Since the descriptors in final MLR models must be independent, the inter-correlations were tested. According to the number of compounds, examined models contained maximum two independent variables. The best one and two parameters equations are summarized in Table 4.

Positive values of regression coefficient indicate that observed descriptor contributes positively to the value of $K_{\text{AM}}/k_m$. Whereas negative values indicate that the greater value of descriptor, the lower value of $K_{\text{AM}}/k_m$.

In case of two tested cationic surfactants strong influences of the electronic descriptors on the retention are noticeable. In order to show molecular descriptors, govern retention in chromatographic system, included CTAB, two parameters models have been applied. It is based on two electronic descriptors, but they are not correlated ($r = 0.121$). Moreover, two parameters model also explains better retention mechanism, when SC as modifier of mobile phase was used. Obtained models present that dipol moment and Dreiding energy plays a crucial role, affecting the retention of macrolide antibiotics in this chromatographic systems.

QSRR analysis showed that only SDS as modifier of mobile phase is useful for the prediction of lipophilicity properties of macrolide antibiotics. The universal scale of lipophilicity is represented by the logarithms of the partition coefficients (log P) between two phases, n-octanol and water in the case of neutral species, or the distribution ratio (log D) for ionizable compound. The traditional direct method for lipophilicity determination (“shake flask” method based on n-octanol-water partitioning) is labor-intensive and time-consuming, so that it is not commonly used nowadays. For this reason, current separation techniques are increasingly used for lipophilicity determination (3). Chromatographic approach has many advantages in comparison to traditional method, it is more convenient, reproducible, faster and inexpensive (4). Additionally, the proposed method for lipophilicity estimation of macrolide antibiotics is attractive from “green chemistry” point of view, because micellar mobile phases are less toxic, nonflammable and have lower environmental impact compared to conventional LC methods. Furthermore, MLC method is faster than previously proposed SOTLC method (17). Although, in SOTLC vapor pressure is lower, because an aqueous solutions of inorganic salts are used as mobile phases.

Table 3. Correlations between and obtained for tested chromatographic systems.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Equation</th>
<th>$r$</th>
<th>$F$</th>
<th>$s$</th>
</tr>
</thead>
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<tr>
<td>SDS</td>
<td>$K_{\text{AM}}/k_m = -0.37 (± 0.038) 1/k_m + 0.037 (± 0.014)$</td>
<td>0.964</td>
<td>91.563</td>
<td>0.0164</td>
</tr>
<tr>
<td>CTAB</td>
<td>$K_{\text{AM}}/k_m = -0.64 (± 0.082) 1/k_m + 0.41 (± 0.018)$</td>
<td>0.948</td>
<td>62.0143</td>
<td>0.0341</td>
</tr>
<tr>
<td>SC</td>
<td>$K_{\text{AM}}/k_m = -0.41 (± 0.039)/k_m - 0.61 (± 0.023)$</td>
<td>0.969</td>
<td>107.526</td>
<td>0.0174</td>
</tr>
</tbody>
</table>

$r$ - the coefficient of correlation; $F$ - the value of Snedecor F-test; $s$ - the standard estimation error; $p$-value $< 0.05; n = 9$.

Table 4. One and two parameters obtained QSRR equations with statistical parameters.

<table>
<thead>
<tr>
<th>$K_{\text{AM}}/k_m$ SDS</th>
<th>$r$</th>
<th>$R^2$</th>
<th>$F$</th>
<th>$s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0.05(± 0.006) \log D_{\text{ACD}} - 0.11(± 0.007)$</td>
<td>0.942</td>
<td>0.888</td>
<td>55.592</td>
<td>0.021</td>
</tr>
<tr>
<td>$0.11(± 0.008) \log D_{\text{ACD}} - 0.02(± 0.003) H_{\text{AM}} - 0.02(± 0.004)$</td>
<td>0.984</td>
<td>0.968</td>
<td>93.106</td>
<td>0.012</td>
</tr>
<tr>
<td>$-0.22(± 0.064) E_{\text{HOMO}} - 2.63(± 1.051) \sigma_{\text{min}} - 9.99(± 2.784)$</td>
<td>0.855</td>
<td>0.731</td>
<td>8.175</td>
<td>0.061</td>
</tr>
<tr>
<td>$-0.02(± 0.006) \mu + 0.99(± 0.047)$</td>
<td>0.762</td>
<td>0.580</td>
<td>9.669</td>
<td>0.047</td>
</tr>
<tr>
<td>$0.0009(± 0.0001) \text{Energy Dreiding} - 0.02(± 0.002) \mu + 0.76(± 0.059)$</td>
<td>0.948</td>
<td>0.898</td>
<td>26.312</td>
<td>0.025</td>
</tr>
</tbody>
</table>

$r$ - the coefficient of correlation; $R^2$ - the coefficient of determination; $F$ - the value of Snedecor F-test; $s$ - the standard estimation error; $p$-value $< 0.05 n = 9$. 

QSAR analysis

The emergence and spread of multiple drug resistance (MDR) bacteria strains, causes that a development of a new antibiotics is nowadays one of the most important challenges of medicinal chem-
Figure 1. Relationship between $1/K_m$ and $K_m/k_m$ for tested chromatographic systems a (CTAB), b (SDS) and c (SC)
istry. Macrolide are broad spectrum antibiotics frequently used to treat infections caused by Gram-positive bacteria, such as respiratory tract and soft-tissue infections. They act by inhibiting protein synthesis by blocking the 50S ribosomal subunit (16). In the early stages of the development of drug candidates, same physicochemical properties of drug candidates such as solubility, lipophilicity, stability and acid–base character should be determined. Since, they can effect on oral absorption, protein binding and metabolism, as well as a pharmacological effect including antimicrobial activity (1). In our previous study, SOTLC was applied in order to find the best SOTLC chromatographic system to predict lipophilicity and biological activity for this class of chemical compounds (17). The antimicrobial activity of antibiotic frequently depends on its lipophilicity (18). However, other chromatographic data, not only chromatographic estimated lipophilicity, can be useful for QSAR study. The data on in vitro antibacterial activity for seven of tested compounds was widely presented in the literature (19). Tylosin is used as veterinary drug, for this reason there are information about its activity for typical bacteria causing animal diseases; Pasteurella multocida, Actinomyces pyogenes, Fusobacterium necrophorum, Brachyspira hyodysenteriae or Mycoplasma hyosynoviae. Furthermore, in the literature are no available data on antibacterial activity of troleandomycin. It is not popular, since this drug is sold only in two countries: Italy and Turkey.

Finally, the minimal inhibitory concentrations (MIC) 50 and 90 of the following bacteria: Staphylococcus aureus, Streptococcus pneumoniae, Listeria monocytogenes, were successfully used for QSAR analysis. Establish QSAR equations are noticed bellow.

Staphylococcus aureus

\[ \text{MIC 50} = 0.25 \pm 0.068 \frac{K_{a0}}{K_m} \text{ CTAB} + 0.16 \pm 0.042 \]

\[ r = 0.853, R^2 = 0.727, F = 13.324, p < 0.014, s = 0.060 n = 7 \]

\[ \text{MIC 50} = -0.39 \pm (0.077) \text{ 1/}K_m \text{ CTAB} + 0.43 \pm 0.047 \]

\[ r = 0.914, R^2 = 0.837, F = 25.619, p < 0.0039 s = 0.068 n = 7 \]

\[ \text{MIC 90} = -0.11 \pm (0.033) \text{ 1/}K_m \text{ CTAB} + 0.50 \pm 0.089 \]

\[ r = 0.832, R^2 = 0.692, F = 11.228, p < 0.02031 s = 0.0933 n = 7 \]

Streptococcus pneumoniae

\[ \text{MIC 50} = -1.89 \pm (0.536) + 0.51 \pm (0.062) \text{ 1/}K_m \text{ SDS} \]

\[ r = 0.844, R^2 = 0.713, F = 12.43, p < 0.01682, s = 0.098 n = 7 \]

Listeria monocytogenes

\[ \text{MIC 90} = 0.06 \pm (0.020) K_{a0}/k_m \text{ CTAB} + 0.18 \pm 0.044 \]

\[ r = 0.809, R^2 = 0.655, F = 9.503, p < 0.0274, s = 0.067 n = 7 \]

\[ \text{MIC 90} = 0.04 \pm 0.012 + 0.75 \pm 0.027 K_{a0}/k_m \text{ SC} \]

\[ r = 0.851, R^2 = 0.725, F = 13.155, p < 0.01511, s = 0.041 n = 7 \]

The results showed, that it is possible to apply chromatographic parameters in order to predict antimicrobial activity of macrolide antibiotics. Similar conclusions can be drawn when we compare obtained results with our previous analyses based on SOTLC. Both SOTLC and MLC chromatographic parameters affect MIC value of Streptococcus pneumoniae, although in case of SOTLC stronger correlations were find. Important might be the fact that in both chromatographic methods retention was highly correlated with lipophilicity.

The significant correlations were found between both MIC values against Staphylococcus aureus and chromatographic parameters obtained on chromatographic system included CTAB in mobile phase. Retention parameters obtained in this chromatographic system were not correlated with lipophilicity of macrolide. This could suggest that antimicrobial activity against Staphylococcus aureus depends on properties of macrolides other than lipophilicity.

Furthermore, chromatographic systems, including CTAB and SC as modifiers of mobile phases, can be used for estimation of antimicrobial activities against L. monocytogenes. The correlation coefficients were close to those observed in our previous study (17).

CONCLUSION

Obtained QSRR equations explain the mechanism of retention and illustrated differences between tested MLC systems. This analysis also confirmed that regression models, which are based on calculated molecular descriptors, can be successfully used for the prediction of chromatographic parameters. Additionally, the study presents the usefulness of chromatographic parameters obtained on different micellar TLC systems on QSAR analysis of macrolide antibiotics. The high correlation coefficients (r), values of F and small values of the standard deviation indicate that the obtained equations can be used for prediction of antimicrobial activity against Staphylococcus aureus, Streptococcus pneumoniae and Listeria monocytogenes. Important is the fact that micellar thin layer chromatography
reduces the time of analysis in comparison to SOTLC. Furthermore, chromatographic system including aqueous solution of SDS may be useful for lipophilicity estimation of those classes of compounds.

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Peloid is described as a maturated mud or muddy dispersion with healing and/or cosmetic properties, composed of a complex mixture of fine-grained natural materials of geologic and/or biologic origins, mineral water or sea water, and common organic compounds of biological metabolic activity (1). The peloids also named as therapeutic muds, thermal muds, clay or ancient peat are known from prehistoric times and were used for healing purposes by Egyptians and Romans (2, 3). Currently, peloids are used in cosmetics and medical treatment in spa resorts. The application of peloids – called pelotherapy is becoming increasingly popular (4).

The use of healing muds in pharmaceutical formulations as gastrointestinal protectors, oral laxatives, anti-diarrheas, dermatological protectors and in esthetic medicine, became more common during last few years due to increasing interest in natural medicine (5, 6). While being selected on empirical bases, all natural materials are not free of possible side-effects (7). Various components of mud, particularly fulvic acid, trace elements may be absorbed through the skin or any skin abrasions, cuts, or other breaches in the integrity of the skin (8-13). Furthermore, after applying the mud directly on the body when it dries, released particles may be inhaled immediately, or they may be incorporated into house dust and enter the respiratory tract at a later time. Accidental mud swallowing by children or a transfer of the material from their hands to their mouths should also be considered. However, there has been no assessment of the potential health risk to consumers posed by toxic elements possibly present in the peloid itself and their toxicity are still lacking (6, 14). In the U.S., the Food and Drug Administration has limited authority over the cosmetics industry and rarely does any routine testing for toxic metals in products used for cosmetic purposes (15, 16). However, FDA regulations state that heavy metal concentrations in cosmetic products should not exceed 10 ppm in the case of Pb. According to European directives, lead is not allowed in cosmetic materials, including clays and peats for pelotherapy (85/391/CEE, 86/179/CEE and 86/199/CEE) (7). Potential toxicity associated with elemental composition of cosmetics is well documented in e.g., eye

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VOLTAMMETRIC DETERMINATION OF TRACE ELEMENTS (Cu, Pb, Zn) IN PELOID-BASED PHARMACEUTICALS

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²Faculty of Materials Science and Ceramics, AGH University of Science and Technology, 30–059 Kraków, al. Mickiewicza 30, Poland

Abstract: The research on the potential health risk posed to consumers by toxic elements that can be found in peloids is still lacking. Moreover, in Polish law no clinical or pharmacological tests are required to identify healing properties of peloids. The objective of this work was to determine some mineral content in selected peloids used in medical treatment. Anodic stripping voltammetry with differential pulse step was used for zinc, copper and lead determination. Decomposition of organic matrix was conducted by a simple wet digestion procedure using acid digestion vessel. Obtained results showed that proposed methods were suitable for the determination of investigated metallic elements. Lead content varied between 0.18 mg/kg (in Mań Borowinowa) and reached up to 15.5 mg/kg of dry weight for Chokrak peloid. Zinc content ranged from 0.64 to 66.87 mg/kg and copper content was between 0.57 and 7.50 mg/kg. The proposed method was validated, the recovery for peloid samples were 94 – 102%; 92 – 97%; 96 – 106% for copper, zinc and lead, respectively.

Keywords: trace element, muds, peloids, medical muds, therapeutic mud

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highlighters (17, 18) muds and clays (6, 10, 19) and other products (20, 21). Moreover, Polish Ministry Regulation in this case defines that peloids should be physico-chemically and microbiologically tested to determine their healing properties, but no clinical or pharmacological tests are required (Dz. U. 2006 Nr 80 No. 565).

Available databases show that spectroscopic methods (ICP-MS, ICP-AES, AAS, XRF) were used for determination of copper, zinc and lead in the majority of available research works (6, 22, 23). All the methods described above, are not free from interferences. The direct determination of metals in samples with high organic matter content by atomic absorption spectrometry is not always possible due to matrix composition. In that case liquid-liquid extraction, flow injection system and solid phase preconcentration techniques were applied (24, 25). The sequential voltammetric procedure allowing determination of zinc, copper and lead was rarely used in biological and environmental samples. Therefore, the application of simple electroanalytical methods should improve analytical performance. The aim of this research was to determine content of zinc, copper and lead in peloids and its products by anodic stripping voltammetry.

**MATERIAL AND METHODS**

**Material**

Artificial clays (peloids) were purchased in drugstore or directly in spa resort. All raw materials were conditioned in room temperature before use. Pasta borowinowa lecznicza – Biochem®, Borowinowa Kostka Iwonicka® from Iwonicz Spa (Poland), Maść borowinowa, Sulphur Zdrój® (Busko Zdrój, Poland), Lecznicza pasta borowinowa®, Kamień Pomorski Spa (Poland) were purchased in drugstore. Chokrak peloid sample was collected from its environment in the Chokrak lake area (Ukraine) in April and May, 2013.

**Apparatus**

A Multipurpose Electrochemical Analyser M161 with a M164 electrode stand (both mtm-anko, Poland) were used for all voltammetric measurements. The standard three-electrode cell consisted of a controlled growth mercury drop electrode (CGMDE) as a working electrode, Ag/AgCl in 3 M KCl with a double junction filled with 3 M KCl (Mineral, Poland) as reference and platinum wire as an auxiliary electrode. Voltammograms were recorded, interpreted and stored by EAGRAPHD 6.0 (mtm-anko, Poland) software.

**Reagents**

A standard solutions of Zn(NO$_3$)$_2$, Cu(NO$_3$)$_2$, and Pb(NO$_3$)$_2$ were prepared by proper dilution of 1 g/L stock standard solution (OUM, Łódź, Poland). Electrolyte used as ionic medium was prepared by dissolving KNO$_3$ (Merck, Suprapur®). For digestion procedures HNO$_3$ (Merck, Suprapur®) and 30% H$_2$O$_2$ (Cheman, Poland) was used. All the solutions were prepared with double-distilled water from quartz distiller (SZ-97A, Chemland, Poland) and all other reagents were of analytical grade.

**Sample preparation**

Samples were homogenized in agate mortar and then dried at over 70°C for 4 hours. Approximately 250 – 500 mg of sample material was accurately weighed and inserted in a Teflon® container of an acid digestion vessel (4748, Parr Instruments Co., USA). Next, the sample was treated with 5 – 6 mL of nitric acid and 1 mL of perhydrol, tightly sealed and was placed in drying oven (Binder, Germany). The digestion of the sample was carried 24 h in 160°C and after it cooled to the room temperature the vessel was unsealed and the sample was quantitatively transferred to an evaporation dish. The digested sample was placed at the heated plate for evaporation and removing the nitrates. The sample solutions were then cooled to room temperature, transferred quantitatively into volumetric flasks (10 mL) and filled up to the mark with double distilled water. All the procedures were carried out in triplicate for each sample.

**Analytical procedure**

The voltammetric procedure for determination of copper, zinc and lead level was in accordance with Table 1. Analytical parameters of voltammetric analysis of selected trace elements.

<table>
<thead>
<tr>
<th>Element</th>
<th>$E_{acc}$ [mV]</th>
<th>$t_{acc}$ [s]</th>
<th>Method</th>
<th>$dE$ [mV]</th>
<th>Working electrode</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(II)</td>
<td>-1200</td>
<td>20</td>
<td>DP ASV</td>
<td>-20</td>
<td>CGMDE</td>
<td>0.2 M KNO$_3$</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>-400</td>
<td>40</td>
<td>DP ASV</td>
<td>-20</td>
<td>CGMDE</td>
<td>0.04 M KNO$_3$</td>
</tr>
<tr>
<td>Pb(II)</td>
<td>-600</td>
<td>40</td>
<td>DP ASV</td>
<td>-20</td>
<td>CGMDE</td>
<td>0.04 M KNO$_3$</td>
</tr>
</tbody>
</table>

$E_{acc}$ - accumulation potential, $t_{acc}$ - accumulation time, $dE$ - pulse amplitude.

Table 1. Analytical parameters of voltammetric analysis of selected trace elements.
Voltammetric determination of trace elements (Cu, Pb, Zn) in... 

... (26). Before each measurement the solution in the voltammetric cell was deaerated by high purity argon for 5 min and then, after redirecting the argon flow over the solution surface, measurements were performed. Zinc, copper, lead were determined using differential pulse anodic stripping voltammetry with a controlled growth mercury drop electrode (CGMDE) with a differential pulse stripping step (27). The measurements were performed according to the standard addition method, voltammograms corresponding to individual additions were registered three times. All experiments were carried out at room temperature. All instrumental parameters of performed measurements were arranged in Table 1.

RESULTS AND DISCUSSION

The proposed voltammetric method using CGMDE after digestion procedure allows determination of zinc (20 s deposition time) with LOD = 0.49 µg/L and LOQ = 1.49 µg/L. The slope for regression line is -2.809 ± 0.036 (nA/µg/L) with correlation coefficients $r = 0.9989$. The recovery of selected peloid samples ranged from 92 to 97% for zinc. The proposed method also allows the determination of copper (40 s deposition time) with LOD = 1.71 µg/L and LOQ = 5.15 µg/L. The slope for regression line is -2.399 ± 0.019 (nA/µg/L) with correlation coefficients $r = 0.9967$. The recovery of selected samples ranged from 94 to 102% for copper. Furthermore, the same method also allows determination of lead (40 s deposition time) with LOD = 0.15 µg/L and LOQ = 0.47 µg/L. The slope for regression line is -1.949 ± 0.036 (nA/µg/L) with correlation coefficients $r = 0.9995$. The recovery of selected peloids ranged from 96 to 106% for lead. For cadmium only LOD and LOQ were estimated at the level 0.09 µg/L and 0.27 µg/L, respectively. The investigated samples showed cadmium level below detection limit and no interpretable signal indicated cadmium presence. During zinc and copper analysis a 1000-fold excess of Fe (III), and 100-fold excess of Pb (II), Cd (II), Mn (II) did not interfere. The surface-active compounds are usually a source of strong interferences in voltammetric methods and should be thoroughly destroyed by digestion prior to analysis.

Zinc showed the highest concentration levels in natural peloid samples, whereas the lowest level values were observed for Pb (II) (Table 2). The highest zinc content was determined in natural samples from Chokrak lake 66.9 mg/kg and the lowest was in pharmaceutical product Borowinowa Kostka lecznicza 0.8 mg/kg. The highest value of copper concentration was determined also in Chokrak lake peloid (4.6 mg/kg) and the lowest was in pharmaceutical ointment MaúÊ borowinowa (0.6 mg/kg). The highest lead contamination was determined in peloid from Chokrak lake 15.5 mg/kg with the smallest moisture. Basing on obtained results positive correlations were observed between the investigated trace elements contents: very high between zinc and lead ($r = 0.86$) and high in pairs Cu-Pb ($r = 0.61$) and Zn-Cu ($r = 0.53$).

The investigated samples were different in origin and a direct comparison is difficult. Among them the Chokrak mud was interesting because it provided a reliable test for the effectiveness of analytical method. Additionally, there are no available data of voltammetric analysis of trace element in peloids. When comparing the results obtained in this study, to the ones from spectroscopic analysis it can

<table>
<thead>
<tr>
<th>Product name</th>
<th>Trace element content (mg/kg d.w.)</th>
<th>Water content [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasta borowinowa lecznicza - Biochem*</td>
<td>Zn(II) 17.6 ± 1.5, Cu(II) 1.6 ± 0.2, Pb(II) 11.6 ± 3.2</td>
<td>92.31</td>
</tr>
<tr>
<td>Borowinowa Kostka Iwonicza* from Iwonicz Spa (Poland)</td>
<td>0.8 ± 0.2, 0.7 ± 0.1, 0.55 ± 0.17</td>
<td>80.26</td>
</tr>
<tr>
<td>Peloid from Chokrak lake (Ukraine)</td>
<td>66.9 ± 4.7, 4.6 ± 0.4, 15.5 ± 3.4</td>
<td>52.27</td>
</tr>
<tr>
<td>Maś borowinowa. Sulphur Zdroj* (Busko Zdroj, Poland)</td>
<td>1.0 ± 0.1, 0.6 ± 0.1, 0.18 ± 0.05</td>
<td>-*</td>
</tr>
<tr>
<td>Lecznicza pasta borowinowa* from Kamień Pomorski Spa (Poland)</td>
<td>20.5 ± 7, 7.5 ± 1.2, 9.6 ± 1.0</td>
<td>80.67</td>
</tr>
</tbody>
</table>

*40 g water extract of peloid/100 g ointment.
be observed that both methods give the same range of concentrations. Chokrak mud zinc and lead content were in the range showed by other works conducted over Portuguese mud where approximately 50 to 150 mg/kg of zinc and 5 to 38 mg/kg of lead were found (6). Moreover, Spanish peloids showed similar zinc and lead concentration with a range between 33.1 - 89.8 mg/kg and 10.9 - 37.5 mg/kg, respectively (23). Peculiarly, according to prior research some peloids can show zinc content up to 160 mg/kg with elevated levels of lead and copper. In comparison, copper level in Chokrak peloid was two times lower than the lowest of previously reported content and in average five to ten times lower than the highest value showed in earlier research (23). However, the distribution of element content was similar showing the following order: Zn > Cu > Pb.

The two types of paste, first from Spa resort and second from market, had similar zinc and lead contents but differed in copper. Correlation between water content and element content were quite low (r = 0.42) mostly due to lead and copper contents. For zinc and water content solely, correlation coefficient showed high value (r = 0.86) but was statistically insignificant. This fact indicates that all the peloids pastes have similar zinc concentration taking into account their water content. According to regulations, the investigated samples can be considered free from heavy metal contamination excluding the peloid from Chokrak lake and Pasta borowinowa lecznicza (Biochem). In this case the lead content was above 10 mg/kg and exceeded the value approved by Polish Pharmacopoeia. However, the limits were exceeded only when calculated for dry sample mass. When water is taken into consideration the contents of lead in the peloid from Chokrak lake and Pasta borowinowa lecznicza – Biochem were 7.39 mg/kg and 0.89 mg/kg, respectively. Moreover, the peloid paste or peloid mud in medical treatment are used in hydrated form directly on the body, so the exceeded limits should not be considered in this case.

CONCLUSIONS

It is necessary to expand study on more samples, but at Polish market there is only a limited number of peloid-based products. However, this study gives an overview on products that are available in Poland and a single, less known peloid from its natural source. The obtained results showed that voltammetric methods used in this study were suitable for determination of copper, zinc and lead both in peloids and derived products. The proposed method is characterized by low instrumental and single analysis costs, ten times lower than the usually applied methods, achieving comparable results with spectroscopic analysis. According to FDA and EC regulations, a trace element content study and analytical quantification for selected elements seem to be necessary, especially when concerning their life important functions and/or their toxicological hazard.

Acknowledgment

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Mushrooms collected in wild are important food items in certain regions of the World and certain species are used also because of medicinal value (1, 2). Demand for edible wild-growing and cultivated fungi in recent decades is growing world-wide (3). Sclerotia is a dense mass of mycelium developed by some saprophytic fungi, which is located under the ground and is collected in some regions of the World as traditional medicine and ingredient in meals (4). Sclerotia of *Wolfiporia extensa* (Peck) Ginns fungus is popular in the Traditional Chinese Medicine – is collected in wild and is cultivated in field condition (2, 5). Database available on biologically active compounds in sclerotia of *W. extensa* is relatively large and many organic constituents have been identified (5, 6), while there is a gap on information about inorganic constituents (7, 8).

Unprocessed fruiting bodies of edible fungi (mushrooms) collected in wild could be rich in an essential macro- and trace inorganic constituents but also at elevated level in certain harmful metallic elements (9). Cadmium (Cd), mercury (Hg) and lead (Pb) are usually considered but some other harmful elements could also matter (10-14).

The province of Yunnan in China is one of the regions in Asia where foraging for sclerotia of *W. extensa* is tradition while cultivation in field condition becomes popular too (5). The Yunnan land, because of the occurrence of the Circum-Pacific Mercuriferous Belt, is one of a specific regions in the World with polymetallic soils that are enriched in Hg, antimony (Sb), barium (Ba), strontium (Sr) and certain other metallic elements (15-17). This can have a consequence in elevated or highly elevated content of Hg in fruiting bodies of certain

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**Abstract:** Dried sclerotia of *Wolfiporia extensa* has a long history of a wide medicinal uses in Asia because of anti-inflammatory effect. There is deficit of information on inorganic constituents of sclerotia. This study aimed at providing and evaluating data on some trace metals (Ag, As, Ba, Cd, Co, Cr, Cs, Cu, Li, Mn, Ni, Pb, Rh, Sr, Ti, U, V, Zn) contents of sclerotia collected across Yunnan land in China. Sclerotia, when compared to fruiting bodies of fungi collected in wild, were low in toxic Ag: range (< 0.005-0.21) mg/kg dry biomass (db); As: (< 0.004-0.041) mg/kg db; Cd: (< 0.002-0.034) mg/kg db; Pb: (0.020-0.24) mg/kg db and Ti: (< 0.001-0.015) mg/kg db. Sclerotia contained detectable amounts of essential trace metals such as Co: (0.011-0.21) mg/kg db; Cu: (0.44-3.8) mg/kg db; Li: (0.005-0.092) mg/kg db; Mn: (2.2-48) mg/kg db and Sr: (0.15-12) mg/kg db. The elements such as Cr, Ni and V occurred in sclerotia in different concentrations, Cr: (0.24-3.3) mg/kg db; Ni: (0.037-14) mg/kg db; V: (0.009-0.25) mg/kg db and U: (< 0.001-0.015) mg/kg db. The sclerotia of *W. extensa* from Yunnan were specifically enriched in Ba and Sr, and content of Mn was similar as showed for fruiting bodies of fungi foraged in Europe. The relatively high contents of Ba and Sr in the sclerotia examined could be attributed to a specific or anomalous geochemistry of soils in the regions of Yunnan.

**Keywords:** medicinal fungi, wild foods, bioconcentration, heavy metal
fungi (mushrooms) foraged in some regions of the Yunnan Province (18-20). Some fungi, e.g. from the genus Agaricus, Boletus, Macrolepiota and others, because of specific genetic features could – even when grown-up at the background areas (without geochemical anomaly and unpolluted) – efficiently accumulate in fruiting bodies harmful Cd, Hg, Ag, and examples of species efficient in accumulation are given by Borovička et al. (21), Falandysz et al. (22), García et al. (23), Gucia et al. (24), Krasińska and Falandysz (25), and Melgar et al. (26).

The fungus Wolfiporia extensa from the family of Polyporaceae grows on the roots of an old, dead pine trees. Sclerotia of W. extensa has attracted considerable attention due to the various medicinal properties, such as anti-inflammatory, sedative, stomachic and diuretic in the Traditional Chinese Medicine for millennia (5, 6). Dried sclerotia is widely used in the form of the decoctions (usually 9 to 18 g of dried powder per day, up to 45 g) and in combination with some other herbs. A traditional snack in Beijing that is called “fuling jiabing” also contains dried sclerotia.

This study is aimed at investigating the occurrence and variability and evaluate data on Ag, As, Ba, Cd, Co, Cr, Cs, Cu, Li, Mn, Ni, Pb, Rb, Sr, TI, U, V, and Zn in sclerotia of W. extensa from poly-metallic soils of Yunnan in China. Elements were determined with inductively coupled plasma – mass spectroscopy and collision cell, what enabled for an efficient elimination of interferences arising from the argon plasma etc. in metal ion detection, and to determine possible variability, associations between the elements and to evaluate the levels.

**MATERIALS AND METHODS**

**Sample collection and initial preparation**

Sclerotia of Wolfiporia extensa (Peck) Ginns were collected from 19 places across of the Yunnan Province in China in July 2012 (Fig. 1). The localizations were selected at an altitude above sea level between 1371 and 2061 m. No specific permits were required for the described field studies. No endangered or protected species were sampled, and the localities where the samples came from are not protected in any way. The inner part of the sclerotia “fuling” which is white in color and free of skin “fuling pi” or pine roots “fu-shen” were separated for a study. From each sclerotia 6 sub-samples of the inner part were taken (each ca. 300 g) and pooled to make a bulk sample. Further, the sclerotia were sliced into small pieces, dried at 105°C and powdered using porcelain mortar. The bulk samples of dry powdered sclerotia (each ca. 600 g) were placed into sealed new polyethylene bags and kept in dry and clean conditions in a herbal materials depository room prior to further analyses.

Figure 1. Localization of the sampling places of W. extensa in Yunnan
Before digestion, the samples were dried at 105°C for 12 h using an electrically heated laboratory oven. Then, subsamples of dried and powdered mushrooms (~0.5000 g) were digested with 5 mL of 65% HNO3 (Suprapure, Merck, Germany) under pressure into the microwave oven model Ethos One (Milestone Srl, Italy). The heating program was performed in one step: the power of the process was 1500 W, ramp time 20 min, temperature 200°C and hold time 30 min. Reagent blank solutions were prepared in the same way. For every set of 10 mushroom samples digested, two blank samples were run. The digest was diluted to 10 mL using deionized water (TKA Smart2Pure, Niederelbert, Germany).

### Sample preparation for analyses

Before digestion, the samples were dried at 105°C for 12 h using an electrically heated laboratory oven. Then, subsamples of dried and powdered mushrooms (~0.5000 g) were digested with 5 mL of 65% HNO3 (Suprapure, Merck, Germany) under pressure into the microwave oven model Ethos One (Milestone Srl, Italy). The heating program was performed in one step: the power of the process was 1500 W, ramp time 20 min, temperature 200°C and hold time 30 min. Reagent blank solutions were prepared in the same way. For every set of 10 mushroom samples digested, two blank samples were run. The digest was diluted to 10 mL using deionized water (TKA Smart2Pure, Niederelbert, Germany).

### Analytical procedure

Elemental analysis of Li, V, Cr, Mn, Co, Ni, Cu, Zn, As, Rh, Sr, Ag, Cd, Ba, Pb, Tl, U was carried out using the ELAN DRC II ICP-MS Inductively Coupled Plasma Mass Spectrometer (PerkinElmer, SCIEX, Canada) equipped with a Meinhard concentric nebulizer, cyclonic spray chamber, collision reaction cell, Pt cones and quadruple mass analyzer. Typical instrument operating conditions for ICP-MS spectrometer were: RF power – 1100 W; plasma Ar flow rate – 15 L/min; nebulizer Ar flow rate – 0.87 L/min and auxiliary Ar flow rate – 1.2 L/min and lens voltage – (7.5-9.0) V. For calibration curves construction, a mixed standard solution with concentration of 10 mg/L was used (Multielement Calibration Standard 3, Atomic Spectroscopy Standard, PerkinElmer Pure). Moreover, the isotopes of 45Sc, 74Ge, 103Rh and 159Tb prepared from individual solutions with concentration of 1000 mg/L were applied as an internal standards in order to effectively correct temporal variations in signal intensity (ICP Standard CertiPUR, Merck, Germany). Calibration curves for elements were constructed in the range of 0.1 to 50 µg/L. Argon with a purity of 99.999% was used as a nebulizer, auxiliary and plasma gas in ICP-MS (Messer, Chorzów, Poland).

### Analytical performance

The methods of trace element measurement was validated and controlled by preparation of standard solutions, calibration of instrument and daily run of blank samples and duplicates and replicates with each analytical cycle. All samples were ana-
lyzed in batches with certified references material and blanks. Duplicates and blanks were measured with every set of 10 mushroom samples. Evaluation of the accuracy of the analytical method was based on analysis of the certified reference material mushrooms powder CS-M-4, Institute of Nuclear Technology and Chemistry in Warsaw, ICHTJ, Poland (Table 1). Precision was calculated as the coefficient of variations (CV) of duplicates. As a result of the analysis, the following measurement precision values were in the range from 1 to 8% for all investigated elements. Finally, the limits of detection, calculated as three standard deviations of 7 independent replicates of the reagent blank, were respectively (in µg/L) for 0.003 for Ag; 0.03 for As; 0.2 for Ba; 0.008 for Cd; 0.001 for Co; 0.2 for Cr; 0.15 for Cu; 0.02 for Li; 0.4 for Mn; 0.05 for Ni; 0.07 for Pb; 0.03 for Rb; 0.06 for Sr; 0.001 for TL; 0.004 for U; 0.09 for V and 4 for Zn.

RESULTS AND DISCUSSION

The data on metallic elements and arsenic contents of sclerotia are summarized in Table 2, and the results from the multivariate examination in Table 3 and in Figure 2. The results presented in this study showed on a highly varying contents of certain elements in sclerotia with the data differing between the places for Ag, Ba, Ni and Sr, low and roughly consistent for Ag, Co, Cs, Li, U and V, and greater and also roughly consistent for Ba, Cr, Cu, Mn, Pb, Rb, Sr and Zn.

Cu, Mn, Zn

Copper, manganese and zinc are essential trace metallic elements both in fungi and fauna, because they are key cofactors in many enzymes. Edible mushrooms foraged in Europe could be considered as good in Cu, Mn and Zn (9). The median value of Cu in examined sclerotia was at 1.3 mg/kg dry biomass (db) and range at 0.085-3.9 mg/kg db. Those values are substantially lower if compared to sclerotia of the fungus named Pleurotus tuber-regium (Fr.) Singer [current name is Lentinus tuber-regium (Fr.)]

![Figure 2. Principal component analysis of the trace metallic elements and arsenic in the Yunnan’s sclerotia of W. extensa](image)
Table 2. Trace metallic elements and arsenic content (mg/kg db) of sclerotia of the fungus *W. extensa* from Yunnan.

<table>
<thead>
<tr>
<th>Place of collection</th>
<th>Li</th>
<th>V</th>
<th>Cr</th>
<th>Mn</th>
<th>Co</th>
<th>Ni</th>
<th>Cu</th>
<th>Zn</th>
<th>As</th>
<th>Rb</th>
<th>Sr</th>
<th>Ag</th>
<th>Cd</th>
<th>Ba</th>
<th>Pb</th>
<th>Cs</th>
<th>Tl</th>
<th>U</th>
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<td>0.016</td>
<td>0.24</td>
<td>9.9</td>
<td>0.022</td>
<td>0.20</td>
<td>3.4</td>
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<td>&lt; 0.002</td>
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<td>0.52</td>
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<td>&lt; 0.0002</td>
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<td>0.37</td>
<td>0.44</td>
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<td>0.015</td>
<td>2.7</td>
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<td>&lt; 0.001</td>
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<td>0.054</td>
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<td>&lt;0.001</td>
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</table>

Notes: *Each value is the mean of 2 digestions and determinations; NA - Not analyzed; Values in italics relate to minimal and maximal content.
Manganese in sclerotia of *W. extensa* was at 9.9 mg/kg db (range 2.9-48) and followed by zinc at 5.1 mg/kg db (range 1.8-9.0) (Table 2). The Mn and Zn contents of the sclerotia of *W. extensa* are much less of *L. tuber-regium* with the manganese median values at 18 mg/kg db and zinc median value at 20 mg/kg db (4).

Fungus *W. extensa* is wood decaying species. Hence, its feeding habit could, to some degree, explain difference in content of Mn and Zn between the sclerotia of *W. extensa* and *L. tuber-regium*. The *L. tuber-regium* is also saprophytic fungus, which is absorbing minerals from a decaying biomass degrading in soils and from soil solution, while usually greater content of Mn and Zn in soils than wood substrata could also matter.

From data published on the trace element composition of mushrooms that are popular in Yunnan could be suggested, that the species substantially vary in contents of Cu and Zn. For example, *Boletus tomentipes* (Earle) Merrill was poor in both elements: Cu was at 11-16 mg/kg db and Zn at 19-23 mg/kg db (28), while much richer was *Boletus edulis* foraged in the central region of Yunnan: range of cooper means value in caps from several places at (17 ± 6) mg/kg db to (880 ± 62) mg/kg db and zinc range was from (54 ± 17) mg/kg db to (530 ± 120) mg/kg db (29).

**Cr, Co, Li**

Chromium (trivalent ion), cobalt and lithium play the statutory roles in human body and are essential, while nutritional requirements for these elements is much lower than for Cu, Mn and Zn. Sclerotia contained Cr at 0.52 mg/kg db (median value), Co at 0.032 mg/kg db and Li at 0.010 mg/kg db. There is no earlier data published on Cr or Li composition of fungal sclerotia. The results for Co in this study are within a range reported for *L. tuber-regium* – with cobalt mean content value at (0.049 ± 0.012) mg/kg db (4).

**Cs, Rb**

Sclerotia contained Cs (133Cs) in range from < 0.001 mg/kg db to 0.043 mg/kg db. Rubidium was at 0.37 mg/kg db (median value), while its minimum was 0.12 mg/kg db and 2.8 mg/kg db was maximum content. No previous data are available on Cs an Rb in sclerotia of *W. extensa*. The sampled sclerotia of *W. extensa* from Yunnan showed very low activity

| Table 3. Factor loadings obtained with unrotated factor matrix. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | PC1             | PC2             | PC3             | PC4             | PC5             |
| **Variables**   | **Li**         | **V**          | **Cr**         | **Mn**         | **Co**         |
| **Eigenvalues** | 6.82           | 3.63           | 2.25           | 1.48           | 1.21           |
| **Total variance (%)** | 37.91           | 20.16           | 12.53           | 8.25           | 6.75           |
| **Li**          | 0.92           | 0.01           | 0.15           | -0.10          | -0.14          |
| **V**           | 0.90           | 0.20           | 0.23           | 0.06           | -0.06          |
| **Cr**          | 0.35           | 0.88           | 0.13           | 0.13           | -0.02          |
| **Mn**          | 0.41           | 0.55           | -0.50          | 0.40           | 0.08           |
| **Co**          | 0.88           | 0.06           | -0.23          | 0.22           | 0.08           |
| **Ni**          | 0.09           | 0.04           | 0.14           | -0.11          | -0.96          |
| **Cu**          | 0.05           | -0.05          | -0.19          | 0.88           | 0.07           |
| **Zn**          | -0.09          | -0.36          | -0.86          | 0.13           | 0.08           |
| **As**          | 0.83           | 0.08           | 0.05           | 0.19           | -0.37          |
| **Rb**          | 0.06           | -0.05          | -0.87          | 0.05           | 0.03           |
| **Sr**          | 0.08           | 0.77           | 0.47           | -0.24          | -0.02          |
| **Ag**          | -0.10          | -0.08          | -0.55          | -0.35          | 0.05           |
| **Cd**          | 0.23           | 0.73           | -0.17          | 0.26           | -0.50          |
| **Ba**          | -0.16          | 0.93           | 0.14           | -0.03          | 0.06           |
| **Pb**          | 0.52           | 0.62           | 0.45           | -0.13          | -0.11          |
| **Cs**          | 0.96           | 0.14           | -0.04          | 0.10           | -0.04          |
| **U**           | 0.94           | 0.10           | -0.04          | 0.08           | 0.11           |
level from radioactive $^{137}$Cs for which the median value was at 3.4 Bq/kg db (7). Sclerotia of $W$.
$extensa$ are remarkably lower in Rb than sclerotia of $L$.
tuber-regium, for which the mean value was at 15 ± 14 (0.05-46) mg/kg db (4).

The elements Rb is common constituents in fruiting bodies. For example in caps of $Amanita$
$muscaria$ Rb was at 58-310 mg/kg db and in $Paxillus$
$involutus$ at 240-480 mg/kg db (30, 31). In the light of data presented, sclerotia of $W$.
$extensa$ from Yunnan are poor in Rb.

$Ba, Sr$

The median value of Ba in sclerotia of $W$.
$extensa$ was at 1.5 mg/kg db and of Sr at 0.68 mg/kg db while for barium range were wide, i.e., from
(0.62 to 22) mg/kg db and strontium from (0.15 to 12) mg/kg db. Those values may suggest on a high
diversity in composition of the geochemical background between the sites sampled. Sclerotia of $L$.
tuber-regium from Nigeria contained Ba at 2.4
(0.045-17) mg/kg db and Sr at 4.3 (0.13-20) mg/kg db (4), which are roughly similar levels.

$Ni, V, U$

The elements Ni and V were at detectable level in all sclerotia sampled. The median value for Ni
was at 0.10 mg/kg db, while maximum content at a single place was as high as 14 mg/kg db (Table 3).
Sclerotia of $W$.
$extensa$ are on the average poorer in Ni than sclerotia of $L$.
tuber-regium, for which median content was at 0.39 mg/kg db, while maximum content was 0.86 mg/kg db (4).
Vanadium also could be detected in all samples of sclerotia but at low level, i.e., median value was at 0.038 mg/kg db, while uranium could not be detected (< 0.001 mg/kg db) with exception of sclerotia from two places (Table 2). There is no data available on occurrence of V and U in sclerotia of fungi other than this study.

$Ag, As, Cd, Pb, Ti$

The elements Ag, As, Cd and Tl could be detected respectively in one to a few samples of sclerotia but at very low level, while element Pb was found in all sclerotia sampled. The median value of Pb in sclerotia was at 0.054 mg/kg db, which is an order of magnitude less when compared to sclerotia of $L$.
tuber-regium – also richer in Ag and Cd (4). Nevertheless, the contents of Ag, Cd and Pb in sclerotia of both species mentioned were, on the average, from two to three orders of magnitude lower when compared to fruiting bodies of popular edible wild-growing fungi (32-34). No data on As and Tl in sclerotia could be obtained from the scientific liter-

Multivariate analysis of data

To examine if any association exits between elements determined in sclerotia has been used a multivariate approach and applying the principal component (PC) analysis (35). We examined the correlation matrix obtained from a possible 19 x 18 data matrix and the results are given in Table 3. The model could explain up to 85.6% variability in the data matrix by five factors for which an eigenvalue was greater than 1. Absolute values of the correlation coefficient were above 0.73 (significance at p < 0.05) for several elements.

The first PC (PC1) was under influence by variables associated with positively correlated As, Co, Cs, Li, U and V. The second PC (PC2) was strongly influenced by positively correlated Ba, Cd, Cr and Sr. The third PC (PC3) was influenced by negatively correlated variables describing Rb and Zn, the fourth (PC4) by positively correlated Cu and Tl, and the fifth (PC5) by negatively correlated for Ni. Figure 2 shows graphically the associations among the elements and sampling sites in the factor space as a PCA.

The trace elements in sclerotia may originate from the wood substrate which provides organic and inorganic compounds to fungus as well as from soil solution which provides water and hence also water soluble compounds – largely inorganic ions but also soluble organic molecules. As can be read from Figure 2A, certain elements tend to cluster together, i.e. Cs, U, Li, V, Co and As (associated with PC1). This configuration of cluster interrelations could be explained by considering that the major source of elements to $W$.
$extensa$ and its host trees in the forested regions of Yunnan is characteristic geo-
chemical composition of soil bedrock and properties of soils of southwestern China but not anthropogenic sources. Those elements at elevated content were specifically in sclerotia from the region of Wenshan in Wenshan country (site 2) and of the Ninglang in the Lijiang area (site 19; Fig. 2C), what strongly suggests an impact from the soil bedrock.
This has been observed recently for Hg and mushrooms foraged in the Yunnan land (18-20). The Ba and Sr, which were positively associated with PC2 usually inter-correlate in fruiting bodies of fungi and this could be related to their chemical analogy with calcium (Ca) that was not measured in this study (36-38). Sclerotia from the region of Jingdong in the Pu’er prefecture (site 4) were specifically enriched in the elements Ba, Sr, Ce and Cd (Fig 2C). With PC4 were associated the Bajiao in the Chuxiong area (site 15) and the Ninglang in the Lijiang (site 19) area due to Cu and Tl (Fig 2D), and with PC5, the Mile in the Honghe area (site 13) due to Ni (Fig. 2D).

CONCLUSION

Dried sclerotia of the fungus *W. extensa* foraged somewhere in the Yunnan Province of China could be enriched in the metallic elements such as Ba, Cr, Sr and Ni. Manganese content of sclerotia is relatively great and similar to levels noted in edible fruiting bodies of wild-growing mushrooms foraged in Europe. The metallic elements such as Co, Cs, Cu, Li, Rb, U, V and Zn could be found in sclerotia but contents are smaller than in fruiting bodies of wild growing mushrooms. The toxic compounds such as Ag, As, Cd, Pb, Rb and Tl are at very low level in sclerotia and without toxicological significance. In future, because of the polymetallic character of a weathered, the red and yellow lateritic soils of Yunnan, any study aiming to provide more data on mineral constituents of sclerotia could focus on a specific areas in this vast in space and rich in forests land, and on associations of fungus with soil geochemistry. Transfer of the elements from a substrate (sclerotia) to edible or of a medicinal use product (decoct etc.) could be also a matter of concern.

Acknowledgment

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Cancer is uncontrolled proliferation of abnormal cells associated with genetic mutation. Despite of all considerable clinical interventions, cancer remains a devastating disease and World Health Organization (WHO) has reported death of 7.6 million people in 2008 (1) and 8.2 million in 2012 (2). Most common cancers include; lungs, breast, liver, stomach and kidneys (3). Incidence rates for cancer is twice as high in developed countries than less developed countries (4). However, mortality rate is high in less developed countries due to lack of health care facilities, poor prognosis and high cost of available treatments. Current therapeutic options for cancer are mainly chemotherapy, radiotherapy, surgery, DNA-interactive agents and hormones (5). However, these treatments are costly, painful and have severe adverse effects. Therefore, there is persistent requirement for new, affordable and effective therapeutic opportunity against cancers. Contemporary health management systems are now preferring medicinal plants for treatment of different ailments (6). In most of the developing countries indigenous medicinal plants are used as folk medicine. Pharmacological evaluation of plants proves them a good source for the medicine development against different infections.

More than 50% of drugs available for cancer therapy are now based on plant derived natural products (7). This is a part of an effort to search new phytochemical based anti-cancer agent with more efficacy, less toxicity and cost effectiveness (8). Moreover, during various studies, phytochemicals have proved to be effective against cell proliferation, apoptosis, metastasis and angiogenic effects (9, 10). However, presently most of them are under clinical trials and only limited number of such natural product based drugs are available for cancer patients (11). In 2005, Chinese Pharmacopoeia listed that petroleum ether, ethyl acetate and methanol extracts of *B. chinensis* root exhibited significant antitumor activity in PC3, Bcap-37 and BGC-823 cells lines (12).

Ornamental plants are mainly grown for their esthetic value but many of them possess lignin, flavonoids and other medicinally important phytochemicals. Some ornamental plants are reported to
have biological activities in various in-vitro and in-vivo studies (13). Sanchezia speciosa (Family: Acanthaceae), is planted as an ornamental plant. Previous reports revealed that methanol extract of Sanchezia speciosa leaves has shown anti-oxidant effects and significant cell growth inhibition on MCF-7 cell line (14). Ethanolic extracts of this plant possess in-vitro cytotoxic, anti-bacterial, anti-fungal and insecticidal activities (15). The aim of present study was to screen phytochemicals and analyze cytotoxic potential of Sanchezia speciosa using Brine shrimp lethality bioassay and MTT cell proliferation assay.

MATERIALS AND METHODS

Plant material
Sanchezia speciosa fresh, mature plants of 183-244 cm height, were collected in July 2015 from Multan and recognized by Dr. Zafar Ullah, Professor of Taxonomy, Department of Botany, Bahauddin Zakariya University, Multan (Voucher specimen: KEW Royal Botanic Gardens K00882617).

Preparation of plant extract
Sanchezia speciosa leaves, bark, wood and roots were shade dried and ground in mill. The pulverized plant material (700 g) was soaked in dichloromethane for 24 h; followed by methanol extraction. Extracts were concentrated by rotary evaporator under reduced pressure. Plant extracts for wood, bark and root in dichloromethane (DCM) were labeled as SSWD, SSBD and SSRD. Whereas, methanol extracts for wood, bark and root were labeled as SSWM, SSBM and SSRM, respectively.

Primary phytochemical screening
For phytochemical analysis, Sanchezia speciosa was subjected to recommended tests for alkaloids, anthraquinones, glycosides, saponins, flavonoids, tannins, terpenes and steroid as described previously (16-18).

Table 1. Preliminary phytochemical screening of Sanchezia speciosa extract.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Qualitative Screening</th>
<th>Result</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>Dragendorff/ Mayer/ Wagner</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>Borntrage/ Modified Borntrage</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>Keller Kelliani</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Ferric Chloride/ Alkaline</td>
<td>-</td>
</tr>
<tr>
<td>Steroids and Terpenoids</td>
<td>Salkowski/ Libermann-Burchard</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Frothing</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>Lead acetate /Ferric chloride</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) Phytoconstituent present; (-) Phytoconstituent absent

Cytotoxicity analysis
Brine shrimp lethality bioassay
Brine shrimp larvae Artemia salina (Leech) are often sensitive to toxic bioactive compounds. Artificial sea water was managed by adding 3.8 g sea salt in 1000 mL water and then filtered. Sea water was poured in a tank, in larger compartment shrimp eggs were added and covered with aluminum foil to darken the surrounding. Brine shrimp larvae had matured in two days. Thereafter, vials were prepared with 5 mL of sea water in each and by using pasture pipette 10 shrimps were placed in respective vial (30 shrimps/dilution) and sustained in illumination. Number of survived shrimps was examined by 3× magnifying glass, results was documented (19) and recorded as IC50 value.

Hela (human epithelial cervical cancer) cell line culture
The Hela cell line was cultured in 10% fetal calf serum with 50 U/mL penicillin and 50 µg/mL streptomycin, placed in humidified incubator with 5% carbon dioxide and at 37°C temperature maintained.

MTT assay
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium) assay was performed to access reduction of tetrazolium to formazan by production of dehydrogenase enzymes in mitochondria of proliferation cells. Hela cells were seeded (on 96 well microtiter plate) with 5 × 10’ – 10’ mL-1 concentration, 200 µL per well total volume maintained and incubated at 37°C. After 24 h, fresh medium was added with different concentrations of plant extracts and control with standard anti-
In vitro cytotoxicity of *Sanchezia speciosa* extracts on... 1391

cancer drug followed incubation for 72 h at 37°C. After incubation, 15 µL of medium was replaced with 150 µL of fresh medium and MTT, respectively, and incubated for 4 h. After that, insoluble formazan was dissolved in 50 µL DMSO and absorbance was taken at 540 nm. IC₅₀ value was calculated for potent cytotoxic plant extracts against Hela cell line. Dose response of potent extract was evaluated at various concentration (6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL), plates were incubated for 72 h, absorbance was measured at 540 nm by ELISA plate reader and results were recorded (20, 21).

Statistical analysis

Results were expressed as the mean ± SEM. One way Anova and T test were applied in different experiments using Graph Pad Prism 6 software.

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**Figure 1.** Cytotoxic effect of *Sanchezia speciosa* crude extracts on Brine shrimps

**Figure 2.** Hela cells survival rate after treatment with *Sanchezia speciosa* extracts

**Sanchezia speciosa** extracts

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**Brine Shrimp Lethality Assay**

**Effect of *Sanchezia speciosa* on HeLa cell line (MTT assay)**
RESULTS

Qualitative screening of *Sanchezia speciosa* for phytochemical evaluation was performed as per recommended protocols. Results illustrate the presence of alkaloids, glycosides, steroids terpenoids and tannins. On the other hand, anthraquinones, flavonoids and saponins were not detected in *Sanchezia speciosa* extract (Table 1). The cytotoxic effect of DCM and methanol extracts of *Sanchezia speciosa* bark and root were evaluated by brine shrimp lethality assay (BSLA). According to present investigation, varying degrees of lethality were observed when exposed to different doses of test samples. Moreover, lethality was found to be directly proportional to concentration of extracts tested (Fig 1). The DCM extract of *S. speciosa* root appeared to have significant cytotoxic potential with IC_{50} value of 2.52 µg/mL comparable to standard anti-cancer drug, etoposide IC_{50} value of 7.46 µg/mL.

To evaluate the effect of *S. speciosa* on cell proliferation assay, growth prospect of cancer cells was considered in MTT assay, with bark and root extract of *S. speciosa* in DCM and methanol solvent respectively. *In-vitro* anti-cancer effect was assessed on Hela cells in 96-well plates and then incubated at 37°C at different daily intervals (n = 3) against absorbance of formazan at 540 nm. The number of viable cells were determined by taking doxorubicin as control with IC_{50} value calculated as 0.1 ± 0.02 µg/mL. Whereas, SSRD found to have chemotherapeutic effect with IC_{50} value 46.7 ± 1.7 µg/mL (Fig 2). Similarly, dose response MTT assay for SSRD was performed at different concentrations indicating maximum chemotherapeutic effect at 100 µg/mL (Fig 3).

DISCUSSION

Natural products are major source of 40% of drugs available in modern medicine system (20). Phytochemical evaluation is foremost step prior to bioactivity investigation, which leads to discovery of novel therapeutic compound. Most of the people in Pakistan rely on folk medicine from diverse flora of country. Plants contain phytochemicals which play vital role in treatment of various infections (22). Phytoconstituents such as triterpenoids, flavonoids, alkaloids and glycosides are reported to be effective in inflammation, pain, infection and cancers. In present study, phytochemical evaluation of *Sanchezia speciosa* revealed presence of alkaloids, glycosides, terpenoids, steroids and saponins (Table 1) while, alkaloids possess cytotoxic effect which is utilized for medicinal purpose (23). Previously, *Sanchezia speciosa* was explored to have significant anti-bacterial, anti-fungal and moderate insecticidal property (24). Moreover, brine shrimp lethality assay is considered as effective bioassay to assess cytotoxic effect of plant material (25, 26). In addition, this significant lethality is accredited to the occurrence of effective cytotoxic phytoconstituents (27). An extract having IC_{50} below 30 µg/mL is generally considered as a potent bioactive extract (23).
In vitro cytotoxicity of Sanchezia speciosa extracts on...

extracts obtained from root of Sanchezia speciosa (SSRD) were found to have potent cytotoxic activity (IC_{50} value 2.52) as compared to standard anti-cancer drug etoposide (IC_{50} value 7.46). This inhibitory effect of extract might be due to cytotoxic compounds present in extract.

MTT rapid colorimetric assay was employed to determine the effect of Sanchezia speciosa wood (SSWD), root (SSRM) (SSRD) and bark (SSBM) (SSBD) on Hela cells proliferation. Meanwhile, formazan product is only formed in viable respiring mitochondria of cells. To recognize in-vitro cell survival of Hela cells, graph was plotted between cells survived and extracts (Fig 3). The results illustrate that SSRD possess chemotherapeutic effect in the period of administration. In a previous study, methanol leaves extract of Sanchezia speciosa have shown anti-oxidant and anti-cancer effects (14). Whereas, no earlier study on wood, root and bark of Sanchezia speciosa was found. In order to investigate the effect of SSRD extract on Hela cell, another experiment was performed with serial dilutions of SSRD extract. The cell viability was significantly reduced, especially at concentration of 100 µg/mL (Fig 3). These findings suggest that Sanchezia speciosa root possesses cytotoxic properties due to its secondary metabolites. However, further fractionation, isolation and bioassay guided studies are indispensable to obtain pure potent anti-cancer compound.

CONCLUSION

Preliminary screening of Sanchezia speciosa revealed the presence of different phytochemicals. Proliferation and survival rate of Hela cells was reduced by Sanchezia speciosa root extract during MTT assay. Moreover, 100 µg/mL concentration of SSRD was found to be most effective in Hela cells. Therefore, Sanchezia speciosa root extract may possess novel potent anti-cancer compound with enhanced efficacy and limited adverse effects. Further, rigorous phytochemical mechanistic and bioassay guided studies are indispensable.

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Diabetes mellitus is still one of the most leading causes of mortality and chronic morbidity in the world. Generally, diabetes is classified into two main types according to the etiological definitions. Type 1 diabetes is caused by destruction of Langerhans islet beta cells, while type 2 diabetes is due to decreased insulin sensitivity which in late stages is also associated with inadequate mass of functional islet cells (1, 2). Although insulin and oral antidiabetic agents are the mainstay of controlling hyperglycemia and management of early onset complications of diabetes, serious late onset complications appear in a large number of patients (3, 4). A more promising approach to the management of diabetes in insulin-dependent patients seems to be transplantation of Langerhans islet cells (5, 6). However, the scarcity of islets donors, graft failure, and issues of ethics are major problems of this approach.

In the last decade several attempts have been made to use stem cells for generating a renewable source of insulin-producing cells (IPCs) (7). From a practical view, an ideal technique for differentiation of stem cells into IPCs should be simple and repeatable in different labs, and should lead to generation of cells that are responsive to both physiological and pharmacological agents. Recently, it has been reported that a simple procedure comprising

EXPOSURE TO FORSKOLIN IMPROVES TRANS-DIFFERENTIATION OF BONE MARROW STROMAL CELLS INTO INSULIN PRODUCING CELLS; GENERATING CELLS WITH BETTER RESPONSES TO PHYSIOLOGICAL AND PHARMACOLOGICAL AGENTS

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Abstract: Several attempts have been made to differentiate stem cells into insulin-producing cells (IPCs) for the treatment of diabetes. Ideally, the differentiation procedures should be simple, repeatable, and must generate cells responsive to physiological and pharmacological stimuli. In this study, we first tested repeatability of a differentiation method for generating IPCs and then tried to improve the method through pharmacological approach to increase capability of IPCs for glucose- and drug-induced insulin secretion. Rat bone marrow stromal cells were trans-differentiated into IPCs by manipulation of the level of glucose, serum and dimethyl sulfoxide (GSD) in culture medium. The effects of addition of nicotinamide (GSD-NA method) and forskolin (GSD-FK method) to differentiation medium on responses of generated IPCs to physiological and pharmacological stimuli were examined. Results demonstrated that the generated IPCs expressed insulin gene and could reduce blood glucose of diabetic mice following intraperitoneal transplantation. When stimulated with glucose as a physiological cue, the IPCs of GSD and GSD-FK methods expressed and secreted insulin in a regulated manner. The expression of insulin gene was also increased in IPCs of GSD-FK method following treatment with glyburide, 3-isobutyl-1-methylxanthine and forskolin. In conclusion, the GSD method is a simple and repeatable procedure for generating IPCs and modification of the method by increasing cell cAMP using forskolin enhances responsiveness of them to physiological and pharmacological agents.

Keywords: differentiation, forskolin, glucose, insulin, nicotinamide, stem cells

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ulation of the level of glucose, serum and dimethyl sulfoxide (GSD method) in culture medium successfully induces trans-differentiation of bone marrow (BM) derived cells into IPCs that secret insulin and reduce blood glucose after transplantation into hyperglycemic mice (8). However, the authors of this report, like others, have not investigated the responsiveness of differentiated insulin producing cells to pharmacological agents.

In this study, we tested the repeatability of GSD method and investigated the capability of IPCs for initiation of insulin secretion in response to physiological (glucose) or pharmacological (glyburide) stimuli and potentiation of glucose-induced insulin secretion using 3-isobutyl-1-methylxanthine (IBMX, a non-selective phosphodiesterase inhibitor) or forskolin, a known adenylyl cyclase activator. Also, we tried to improve this technique by the addition of nicotinamide or forskolin to the differentiation medium. Nicotinamide, the amide derivative of nicotinic acid, is poly (ADP-ribose) polymerase inhibitor. It has been shown that nicotinamide can induce beta cell outgrowth from undifferentiated fetal pancreatic cells (9), stimulates differentiation of embryonic stem cells into insulin-secreting cells (10-12) and enhances insulin production in fetal pig IPCs (13). Forskolin is known to alter the lineage commitment of BM stromal cells through activation of cAMP signaling pathway (14). The cAMP plays an important role in cell proliferation and differentiation of progenitor cells and has been suggested to promote beta cell survival (15-17). It was found here that the addition of forskolin to the differentiation media generates IPCs which are more responsive to the pharmacological interventions.

EXPERIMENTAL

Chemical compounds

Low glucose (5.5 mM) and high glucose (25 mM) Dulbecco’s Modified Eagles Medium (DMEM), trypsin, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), penicillin-streptomycin solution, and dimethyl sulfoxide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Streptozotocin was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Rat insulin RIA kit was obtained from Izotope (Budapest, Hungary). Total RNA purification kit was purchased from Jena Bioscience (Germany). The RevertAid first strand cDNA synthesis kit was supplied from Thermo Scientific (Pittsburgh, PA, USA). Taq polymerase, dNTP, 10x buffer were obtained from Cinagen Company (Tehran, Iran).

Animals

Male Wistar rats (180-200 g, 10-12 weeks old) and male Bulb/C mice (26-30 g, 10-12 weeks old) were obtained from Laboratory Animals Research Center, Mashhad University of Medical Sciences. The animals were maintained in the laboratory in room temperature (22 ± 2°C) under a 12 h light/dark cycle. They had free access to normal laboratory chow and tap water ad libitum. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Mashhad University of Medical Sciences.

Preparation of collagen-coated coverslips

Tail tendon fibers were taken from male rats and sterilized under UV light for 24 h. Then, the fibers were suspended in 0.1% acetic acid and stirred at 4°C for 48 h. The mixture was left at 4°C for 24 h without agitation allowing the undissolved fibers to sediment. Upper clear solution was separated as collagen solution. The slide coverslips (22 × 22 cm²) were sterilized by autoclave and immersed in 0.3% collagen solution for 48 h at 4°C. At the end of this period, the coverslips were dried under laminar hood and then decacidified by a solution of phenol red in phosphate buffer. The collagen-coated coverslips were placed in wells of six-well tissue culture plates and sterilized under UV light for 24 h.

Preparation of BM cells

The BM stromal cells were isolated from femur and tibia of rats. The animals were sacrificed with high ether anesthesia and with aseptic technique the bones of two hind limbs were removed. The proximal and distal heads of bones were cut, and the marrows were harvested by flushing medullary cavities with low glucose (5.5 mM) DMEM. The BM cells were filtered through a blood set filter (pore size 200 µm) to remove debris and bone fragments.

Differentiation of BM cells into IPCs

In GSD method, the BM stromal cells were seeded onto collagen-coated coverslips in six-well culture plates (1.5 × 10⁵ cells/well) containing serum-free low glucose (5.5 mM) DMEM supplemented with 1% DMSO. After 3 days culture, the medium was changed by high glucose (25 mM) DMEM containing 10% FBS. The cells were incubated in this medium for 7 days, while the medium
was replaced every 3 days. In separate experiments, after 3 days culture in the above mentioned medium, the cells were incubated for 7 days in high glucose DMEM containing 10% FBS plus 10 mM nicotineamide (GSD-NA method) or 1 µM forskolin (GSD-FK method).

**PCR test**

In order to approve that the IPCs have transdifferentiated into endocrine hormone-producing cells, the expression of insulin-1, somatostatin and glucagon genes was evaluated by RT-PCR. Total RNA was extracted using RNA purification kit and cDNA was synthesized according to the manufacturer’s instruction. Then, PCR was performed using specific primers mentioned in Table 1. For the mentioned genes, PCR amplification was carried out in 20 µL reaction volumes containing 40 ng cDNA, 0.25 mM dNTPs, 2.5 mM MgCl₂, 1X reaction buffer, 0.5 µM of each primers, and 0.4 units of Taq polymerase. PCR experiment was performed under the following conditions for all three genes: 95°C for 5 min, followed by 35 cycles at 95°C for 45 seconds, 58°C for 40 seconds, 72°C for 1 min, and finally a 7 min extension at 72°C. Finally, the amplified products were carried out using Veriti® Thermal Cycler – Applied Biosystems (USA) and all PCR products were analyzed using 2% agarose gel. Results were visualized under UV light by a GelDoc system.

**Transplantation of IPCs**

The repeatability of GSD method for controlling blood glucose following transplantation of IPCs was investigated in diabetic mice. Diabetes was induced by intraperitoneal injection of 160 mg/kg streptozotocin. At day 10 after streptozotocin injection, mice were randomly divided into two groups (n = 4): diabetic transplant group that received an intraperitoneal transplant of 100 µL IPCs (10⁵ cells/100 µL) suspension in saline, and diabetic control group that received 100 µL saline solutions as vehicle. The animals were considered to be diabetic if they had fasting blood glucose level of 300 mg/dL or higher (18) at day of transplantation. Blood glucose was measured using a standard blood glucose meter (Roche Diagnostics).

**Evaluation of physiological responses of IPCs**

Physiological responses to different concentrations of glucose were investigated in IPCs trans-differentiated with GSD, GSD-NA, and GSD-FK methods. The IPCs were pre-incubated for 24 h in low glucose DMEM containing 10% FBS. Then, to specifically measure insulin release without interference from the FBS, the IPCs were incubated in serum-free media containing 5.5, 10 or 20 mM glucose. After 2 h of incubation, the conditioned media were collected and stored at -20°C until insulin assay. Insulin-1 concentration in the culture media was measured by an immunoradiometric assay. To evaluate the effect of glucose on the level of insulin expression, similarly the IPCs were incubated overnight in the media containing 5.5, 10 or 20 mM glucose.

**Evaluation of pharmacological responses of IPCs**

Pharmacological responses to IBMX, forskolin and glyburide were investigated in IPCs trans-differentiated with GSD, GSD-NA, and GSD-FK methods. The IPCs were pre-incubated for 24 h in low glucose DMEM containing 10% FBS. Then, the IPCs were incubated in serum-free media containing 10 mM glucose in the absence or presence of 100 µM IBMX, 10 µM forskolin or 10 µM glyburide. After 2 h of incubation, the conditioned media were collected for insulin assay. To evaluate the effect of IBMX, forskolin and glyburide on the level of insulin-1

<table>
<thead>
<tr>
<th>Table 1. Sequence of primers.</th>
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<tbody>
<tr>
<td><strong>Gene</strong></td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>Insulin-1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
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<td></td>
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<td>GAPDH</td>
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expression, the IPCs were incubated overnight in the media containing 10 mM glucose and test drugs.

**Quantitative real-time PCR**

The mRNA expression level of insulin-1 was evaluated by Real time PCR. After total RNA extraction and cDNA synthesis, PCR was performed using specific primers mentioned in Table 1. Real time PCR was carried out by SYBR green method. All reactions were performed in triplicate and glyceraldehyde phosphodehydrogenase (GAPDH) was used as reference gene. Expression level changes were estimated by $\Delta\Delta CT$ method.

![Figure 1. Morphological changes of BM stromal cells trans-differentiated into IPCs with GSD method. A: Undifferentiated cells cultured 10 days in control medium; B: the BM cells underwent morphological changes into more rounded shapes and generated cell clusters under differentiated medium. Scale bars, 50 µm](image)

![Figure 2. Expression of genes related to pancreatic islets cells trans-differentiated from BM stromal cells with GSD method. RT-PCR was performed to detect insulin-1, glucagon and somatostatin](image)
Exposure to forskolin improves trans-differentiation of bone marrow stromal cells into insulin...

Figure 3. Glucose change of diabetic mice after intraperitoneal transplantation of IPCs trans-differentiated from BM stromal cells. Diabetes was induced by intraperitoneal injection of streptozotocin at day 1. *p < 0.05, **p < 0.001 compared with the values of day 1 in the corresponding group. *p < 0.05, **p < 0.01 compared with diabetic transplants at the corresponding day. Each value represents mean ± SEM (n = 4).

Figure 4. Effect of different concentrations of glucose on the release of insulin by IPCs. Rat BM stromal cells were trans-differentiated into IPCs by manipulation of the level of glucose, serum and dimethyl sulfoxide (GSD) in culture medium, in the absence or presence of nicotinamide (GSD-NA method) or forskolin (GSD-FK method). The IPCs were incubated in serum-free media containing 5.5, 10 or 20 mM glucose for 2 h. *p < 0.05, **p < 0.01 compared with glucose of 5.5 mM in the corresponding group. ***p < 0.01 compared with glucose of 5.5 mM in GSD group. Each value represents mean ± SEM (n = 4).
Statistical analysis

Statistical analyses were performed using Student’s t-test, when there were two groups to be compared and one-way analysis of variance followed by Tukey’s post hoc test, when there were more than two groups. All experiments were repeated at least four times. Results are expressed as the mean ± standard error of the mean (SEM) and the p-value less than 0.05 was considered to be statistically significant.

RESULTS

Morphological changes and gene expression of BM-derived IPCs

Figure 1A shows BM-derived stromal cells after day 10 culture in control condition. The cells were spindle shaped with long projection and could proliferate in vitro. On the other hand, the cells cultured for 10 days in differentiation medium with GSD protocol underwent morphological changes into more rounded shapes and generated cell clusters look like islet structure (Fig. 1B). The islet-like clusters are stable for at least 4 weeks in culture medium.

To confirm BM cells have undergone pancreatic differentiation, gene expression profiles related to pancreatic islet cells were assessed using RT-PCR analysis. Figure 2 shows the expressions of insulin-1, glucagon, and somatostatin in cells trans-differentiated with GSD method. While all of the three genes were expressed in the differentiated cells, no expressions were detected in control (undifferentiated) cells.

Glycemic status in diabetic mice following transplantation of IPCs

In diabetic control rats, the level of fasting blood glucose continually increased from day 1 to 30 post-diabetes induction (Fig. 3). The animals receiving intraperitoneal transplant of IPCs began to decrease their blood glucose within one week. At day 7 and 20 after cell therapy, the level of blood glucose in treated group was significantly lower than that in control group (p < 0.05).

Physiological responses of IPCs to glucose level

In IPCs trans-differentiated with GSD method, the release of insulin in the presence of 5.5 mM, 10 mM and 20 mM glucose was 36 ± 1.5 pg/ml, 69 ± 4 pg/mL (p < 0.01 vs. 5.5 mM) and 92 ± 10 pg/mL (p < 0.01 vs. 5.5 mM), respectively (Fig. 4). Similarly, the release of insulin was significantly increased by enhancing glucose concentration of incubation.
Exposure to forskolin improves trans-differentiation of bone marrow stromal cells into insulin...

media in IPCs trans-differentiated with GSD-FK technique. In IPCs of GSD-NA method, the increase of insulin secretion in response to 10 mM glucose was comparable to those of GSD and GSD-FK techniques (p < 0.01 vs. 5.5 mM); however, no further increase was observed in the presence of 20 mM glucose. Comparison between methods showed that the level of basal insulin release (in the presence of 5.5 mM glucose) by IPCs trans-differentiated with GSD-FK and GSD-NA methods is higher than GSD technique, while their response to 10 mM and 20 mM glucose was not significantly different among the three methods.

The data of quantitative real-time PCR demonstrated that, in glucose concentration of 10 mM, insulin-1 gene expression was significantly (p < 0.05) higher in IPCs generated by GSD-NA method than those of GSD method (Fig. 5). Increase of glucose concentration in the culture medium from 10 mM to 20 mM led to a significant increase in expression of insulin gene in IPCs of GSD (p < 0.05) and GSD-FK (p < 0.01) methods, but not in IPCs of GSD-NA method. The IPCs generated by GSD-FK method showed the highest insulin gene expression in the presence of 20 mM glucose.

Pharmacological responses of IPCs to IBMX, forskolin and glyburide

As shown in Figure 6, 2 h incubation with 10 µM glyburide led to a nonsignificant increase in insulin secretion by IPCs of all GSD, GSD-FK and GSD-NA methods. Similarly, incubation with 10 µM forskolin increased the secretion of insulin by IPCs trans-differentiated with either the three methods, however the level of increase was not statistically significant. Regarding IBMX, the IPCs trans-differentiated with GSD-FK were the only IPCs that showed significant pharmacological responses. In the presence of 100 µM IBMX, insulin released to the culture media increased from 74 ± 3.6 pg/mL to 96 ± 6 pg/mL (p < 0.05).

Quantitative real-time PCR demonstrated that the expression of insulin-1 was not significantly altered in IPCs of GSD method as a result of glyburide or forskolin treatment (Fig. 7). Only IBMX was able to enhance (p < 0.05) insulin gene expression in IPCs trans-differentiated with GSD method. In IPCs generated by GSD-NA method, forskolin significantly decreased insulin expression (p < 0.05) while glyburide and IBMX were ineffective. On the other hand, the level of transcript insulin was increased significantly in the IPCs of GSD-FK method as a result of glyburide (p < 0.05), forskolin (p < 0.05) or IBMX (p < 0.01) treatment.

DISCUSSION AND CONCLUSION

Utilization of stem cells for generating a renewable source of pancreatic islet cells for transplantation to diabetic patients has become the subject of intense research over the past years (8, 19-21). Stem cells can be obtained from embryos, fetuses, and adults (usually from BM, adipose tissue, teeth, skin, and umbilical cord blood) (22-24). There are reports that stem cells harvested from BM, adipose tissue, pancreas, skin, and embryos have the potential to differentiate into endocrine cells capable of releasing islet hormones (19, 20, 25-27). From a practical view, differentiation techniques for generating islet cells should be simple, repeatable and ethically justifiable, and the generated IPCs should possess physiological secretory machinery in terms of
glucose-induced insulin release which responds to insulinotropic agents. From the legal and ethical point of view, the use of adult sources for cell therapy appears to raise no new issues and is preferable to the use of embryos or fetuses sources (27). After an extensive review of literature on techniques for differentiation of adult stem cells into IPCs, it was found that the method of GSD described by Oh et al. is a simple, rapid, and inexpensive technique (8). In the present work, our results on gene expression, insulin release, and transplantation are in accordance with those of Oh et al. and therefore confirm the repeatability of GSD method. Then, we tried to improve the method of Oh and coworkers by the addition of some factors, which are reported to influence beta cell mass, to the differentiation medium.

Until now, several factors have been identified that influence beta cell mass in the pancreas. These include hormones, glycemic status, growth factors, and phytochemical ingredients (28-31). In the past years, some attempts have been made to use these factors for increasing islet mass in vivo or improving methods of beta cells differentiation in vitro. For example, nicotinamide has been reported to stimulate differentiation of embryonic stem cells into IPCs (9-12). Also, glucagon-like peptide-1 (GLP-1) and exendin-4, a long-acting GLP-1 receptor agonist, have been shown to enhance regeneration of beta cell mass in diabetic animals (32). Incubation of fetal human islet cells with GLP-1 caused maturation of the beta cells (33). In neonatal rat islets, a three-fold increase in the number of beta cells was seen following 24 h incubation with GLP-1 (31). In our study, it was found that supplementation of differentiation medium of GSD method with nicotinamide improves glucose sensitivity of IPCs, and supplementation with forskolin generates IPCs which are more responsive to both glucose and pharmacological insulinotropic agents.

Insulin secretion from beta cells is initiated by membrane depolarization which is regulated by ATP-dependent potassium (K_{ATP}) channels and augmented by protein kinase A and protein kinase C dependent signaling pathways. Glucose increases insulin secretion through increasing the ratio of ATP/ADP and closing the K_{ATP} channels, and its extracellular concentration is the major physiologic controller of insulin secretion (34). Forskolin and IBMX increase intracellular cAMP by stimulating adenylyl cyclase and inhibiting cyclic nucleotide phosphodiesterases (PDEs), respectively, and therefore potentiate glucose-induced insulin secretion (35, 36). On the other hand, glyburide, a sulfonylurea hypoglycemic agent, elicits insulin release by closing K_{ATP} channels (34). In our study, to investigate the physiological capability of IPCs, we assessed their glucose-induced insulin release. It was found that insulin release by IPCs of all differentiation methods was significantly increased in response to glucose enhancement in a concentration-dependent manner. It seems that forskolin and nicotinamide improves the sensitivity of the IPCs to glucose as evidenced by increasing insulin release in 5.5 and 10 mM glucose while the maximum of glucose-induced insulin secretion was not changed. We may conclude that in the modified methods glucose response curve was shifted to the left resulting in reduction of glucose EC_{50}. This finding is in agreement with previous evidence which showed CAMP-dependent signal transduction has an important role in susceptibility of beta cells to glucose (35, 36).

Glucose controls insulin gene expression through recruitment of several transcription factors including mammalian homologue of avian MafA/L-
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Maf, pancreatic/duodenal homeobox-1, and Beta2/Neuro D (37, 38). Accordingly, our results showed that the expression of insulin gene increased in the IPCs of GSD and GSD-FK methods following glucose increase. This effect was especially obvious in IPCs of GSD-FK method. On the other hand, the IPCs of GSD-NA method showed the highest level of insulin gene expression in glucose level of 10 mM.

It has been shown that both cAMP and nicotinamide augment the glucose-activated insulin gene transcription, but with different mechanism. While, cAMP stimulates insulin gene transcription through cAMP response elements, nicotinamide enhances insulin biosynthesis, in part, through increased MafA gene transcription (39, 40). These data indicate that continuous stimulation of MafA and cAMP-dependent pathway during differentiation of progenitor cells leads to IPCs that have better response to physiological and pharmacological cues.

Regarding response to pharmacological insulinotropic agents, the IPCs of GSD-FK method were the only IPCs that showed increased insulin release in response to IBMX, and demonstrated increased insulin expression in response to all glyburide, forskolin and IBMX. These data demonstrate that stimulation of cAMP pathway during differentiation leads to IPCs that also have better response to pharmacological cues. It has been shown that elevation of intracellular cAMP content stimulates the transcription of cyclic nucleotide phosphodiesterases (PDEs) (40). A previous study showed that PDEs have important role in secretion of insulin from isolated pancreatic islets (41). The better insulin secretion in IPCs of GSD-FK method may be the result of higher activity of PDEs which implicates an active role for PDEs in glucose-induced insulin release.

In conclusion, our data indicate that the GSD method is a simple and repeatable procedure for generating IPCs and use of forskolin improves this method so that enhances glucose-induced insulin secretion and responsiveness of these cells to pharmacological initiators of insulin secretion (such as glyburide) and to potentiator of glucose-induced insulin secretion (IBMX, and forskolin). Keeping in mind the growing demand for IPCs transplants, further studies are warranted for optimizing protocols of generating these cells from stem cells.

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Cancer, diabetes and hemophilia are caused by the over or under production of proteins and mutated proteins (1). The genetic material that codes for proteins production is DNA, so extensive research is being carried out in order to understand the interactions of drug molecules with DNA (2). Many features of the rich literature insighting drug binding to DNA have been studied in recent articles, symposia and books (3-5). There are different modes of interaction of drug with DNA but the molecules which are planar and hydrophobic in nature tend to have ability to bind with DNA via intercalative mode of interaction. This mode of binding is also confirmed by the theoretical studies and molecular docking shows this mode clearly in the different conformer for the ligand (6).

Heterocyclic compounds play an important role in an untiring effort aimed at developing new chemotherapeutic agents with new mechanism of action (7). Among them, conjugated heterocycles derived from 1,2,4-triazoles have received considerable attention due to the synthetic and effective biological prominence. A large number of ring systems containing 1,2,4-triazoles have been incorporated into a wide variety of therapeutically interesting drug candidates (8, 9). 4-Amino-3-substituted-5-mercapto-1,2,4-triazole ring systems (10).
The need for privileged scaffolds in medicinal research gives an impetus for the synthesis of hybrid structures with remarkable activity profile and in a continuation of an ongoing program aiming at finding new structural leads with potential chemotherapeutic applications (11-15), it was rationalized to emphasize on a structural library of triazolothiadiazines in combination with DNA binding affinities calculated by the voltammetric measurements (16-18). The computer-aided molecular docking study was also performed in this work to evaluate the binding properties which affords valuable information of drug binding mode in the active site of DNA and may lead to the rational design of new class of anticancer drugs (6, 19).

RESULTS AND DISCUSSION

Synthetic pathway adopted for the synthesis of 1,2,4-triazolo[3,4-b][1,3,4]thiadiazine derivatives (6a-e) is sketched in Scheme 1. Ethyl 4-methoxybenzoate (2) was synthesized by the acid-catalyzed esterification of 4-methoxybenzoic acid (1) in absolute ethanol. The ester (2) was converted into corresponding aromatic acid hydrazide (3) by refluxing with hydrazine hydrate (80%) in ethanol. The 4-methoxybenzohydrazide (3), on reaction with carbon disulfide in ethanolic potassium hydroxide afforded corresponding dithiocarbazinate (4) in good yield and was directly used for the next step without further purification. 4-Amino-5-(4-methoxyphenyl)-4H-1,2,4-triazole-3-thiol (5) was synthesized by refluxing 4 with hydrazine hydrate (80%). Condensation of triazole (5) with various phenacyl bromides in absolute ethanol under reflux conditions afforded 1,2,4-triazolo[3,4-b][1,3,4]thiadiazines (6a-e) in good yield. The spectro-analytical data of these compounds are reported in our recently published report (20).

Voltammetric studies of the interaction of compounds (6a-e) with DNA

The cyclic voltammetric measurements were carried out in order to understand the redox behavior and the DNA binding affinities of the newly prepared compounds (6a-e). The cyclic voltammograms of 6a-e showed only one broad cathodic peak that is reduction peak, there is no oxidation peak which reflects the irreversible nature of the system. The absence of oxidation peak during reverse scan revealed the unwavering reduced form of compounds 6a-e.

<table>
<thead>
<tr>
<th>Entry</th>
<th>ΔE/V</th>
<th>Formation constant $K_f$ (M$^{-1}$)</th>
<th>$\Delta$G (kJ/M) Experimental</th>
<th>$\Delta$G (kJ/M) Theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a</td>
<td>0.0288</td>
<td>5.00</td>
<td>-32.51</td>
<td>-34.31</td>
</tr>
<tr>
<td>6b</td>
<td>0.0671</td>
<td>2.50</td>
<td>-30.79</td>
<td>-32.75</td>
</tr>
<tr>
<td>6c</td>
<td>0.0198</td>
<td>4.80</td>
<td>-32.41</td>
<td>-33.87</td>
</tr>
<tr>
<td>6d</td>
<td>0.0158</td>
<td>1.18</td>
<td>-28.93</td>
<td>-31.33</td>
</tr>
<tr>
<td>6e</td>
<td>0.0231</td>
<td>3.25</td>
<td>-31.44</td>
<td>-33.72</td>
</tr>
</tbody>
</table>

Scheme 1. Synthetic scheme for triazolothiadiazines (6a-e)
For irreversible system, the number of electron transfer in the electrochemical process (21) can be determined by the following equation:

$$|E_{p} - E_{1/2}| = 47.7/\alpha n \text{ mV}$$

(1)

where $\alpha$ is the transfer coefficient and $n$ is the number of electron transfer.

For every compound it had been calculated that the number of electron transfer in each system was $\approx 1$. The addition of 0.77, 1.12, 1.54 and 2.7 µM DNA to 1 mM concentration of the compounds (Fig. 2), showed a decrease in cathodic peak current and positive shift in peak potential. The variation in peak current and shift in potential of compounds (6a-e) was used to calculate formation constant $K_f$ and mode of interaction with DNA. The positive shift in peak potential is attributed to the intercalative mode of the 6a-e into the double helix strands of DNA (22) whereas the decrease in peak current can be explained as, there is formation of drug-DNA adduct due to which free drug concentration decreases as a result peak current decreases. This gradual decrease in peak current of drugs by addition of increasing concentration of DNA was probed to calculate the formation constant $K_f$ of drug-DNA adduct (23) by using equation 2 and is listed in Table 1.

$$I_{p2} = \frac{1}{K_f[DNA]} (I_{p2} - I_{p2}^0) + I_{p2}^0$$

(2)

where $I_{p2}$ and $I_{p2}^0$ is peak current with and without DNA, $K_f$ is the formation constant and [DNA] is the concentration of DNA used. By plotting $I_{p2}$ vs. $(I_{p2} - I_{p2}^0)/[DNA]$, we get the slope value and the inverse of slope gives the value of formation constant $K_f$.

From the data listed in Table 1, the values of formation constant $K_f$ obtained for all compounds are impressive having order of $10^5$. The greater binding affinity is revealed by compounds 6a and 6c substituted with chloro and fluoro groups whereas compound 6d possessing a naphthyl group shows the least binding potency. The increase in steric hindrance may well be the responsible factor for the decrease in binding affinity and it can also be justified by the introduction of another chloro sub-

---

Table 2. Data from PyRx for ligand 6a (6 different conformers and their respective free energy and slandered deviation values).

<table>
<thead>
<tr>
<th>Ligand-DNA</th>
<th>Binding affinity kJ/M</th>
<th>RMSD/upper bound</th>
<th>RMSD/lower bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-6a (1)</td>
<td>-34.317</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DNA-6a (2)</td>
<td>-34.317</td>
<td>12.723</td>
<td>8.397</td>
</tr>
<tr>
<td>DNA-6a (3)</td>
<td>-33.0615</td>
<td>15.681</td>
<td>12.55</td>
</tr>
<tr>
<td>DNA-6a (4)</td>
<td>-32.2245</td>
<td>7.264</td>
<td>4.965</td>
</tr>
<tr>
<td>DNA-6a (5)</td>
<td>-33.0615</td>
<td>15.806</td>
<td>11.558</td>
</tr>
<tr>
<td>DNA-6a (6)</td>
<td>-31.0615</td>
<td>14.860</td>
<td>11.509</td>
</tr>
</tbody>
</table>

---

Figure 1. Cyclic voltammograms of 20% aqueous DMSO buffer at pH 6.0 (no peak); supporting electrolyte 0.1 M TBAP and 0.1M 6a, showing strong reduction peak in selected potential window.
constituent (6e), the value of formation constant decreases which may also be due to the same reason i.e., steric hindrance. But having values of formation constant \( K_f \) in order of \( 10^5 \) make us to think that these compounds prove to have value as DNA binder.

The DNA binding potency of (6a and 6c) are greater than epirubicin (\( K_f = 4.1 \times 10^4 \) M\(^{-1}\)), the clinically used anticancer drug (24). The binding free energy change \( (\Delta G = -RT\ln K) \) of synthesized compounds in KJ/M at 25°C signifies the spontaneity of drug-DNA interaction and the high value of \( \Delta G \) also justifies the high conformational stability of compound-DNA complex (25).

**Docking studies**

Theoretically different software like autodock vina and PyRx are very useful and give very interesting information about the drug-DNA interaction. By using these computational studies, we can pre-
dict and design some new drugs without wet lab (19, 26). The behavior for these compounds shows some strong interaction and from different conformers, we observe the mechanism which is mostly intercalation (Fig. 3). Different conformers after results analysis in PyRx that is supported by vina and autodock show mechanism of binding and also tabulated results for free energy.

With the same structure for DNA ligand has different probabilities to bind with intercalation mode. Free energy values from dockings are approximately equal to the experimental values and fully support the experimental data as represented by compound 6a in Table 2. We use the minimum slandered deviation values to compare and in Table 2, it’s the first one. Furthermore, docking studies of the synthesized compounds confirmed both the qualitative as well as quantitative results.

**EXPERIMENTAL**

**Synthesis**

The compounds were synthesized according to our recently published report (20).

**DNA binding studies**

Sodium salt of DNA (Acros) was used as received. The concentration of DNA stock solution was calculated by measuring UV absorbance at 260 nm using molar extinction coefficient (ε) value 6600 M⁻¹/cm whereas the ratio of absorbance at 260 and 280 nm indicated that the DNA is free from protein (27). Cyclic voltammetric (CV) measurements were performed with the objective of getting insight into redox behavior and DNA binding potency of compounds (6a-e) in a single compartment cell with a three electrode configuration by using Eco Chemie Auto lab PGSTAT 12 potentiostat/galvanostat.
(Utrecht, The Netherlands) with the electrochemical software package GPES 4.9. The three electrode system consisted of reference electrode: Standard Calomel Electrode SCE (Fisher Scientific Company cat # 136395), a Beckman platinum wire of thickness 0.5 mm with an exposed end of 10 mm as the counter electrode and a bare glassy carbon electrode (surface area of 0.071 cm$^2$) as working electrode. Prior to experiments, the voltammogram of a known volume of the solvent system was recorded in the absence of compound and DNA after flushing out oxygen via purging argon gas for 10 min. The procedure was then repeated for systems with constant concentration of the drug and varying concentration of DNA. The working electrode was cleaned after every electrochemical assay.

Molecular docking

Molecular structures for the ligands were drawn on the chemsk11 and then geometrically optimized by the HyperChem8. Autodock4 and autogrid4 run in PyRx-0.8 docking software which automatically generates the PDBQT files for both ligand and macromolecule. Selection of the appropriate grid PyRx run its calculation and gives the results in tabulated form.

CONCLUSIONS

In summary, this work demonstrates the successful synthesis of a series of condensed heterocycles; 3,6-disubstituted-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine derivatives which show higher DNA binding affinities calculated from the voltammetric measurements. These results unfailingly open up the binding mode and interaction strength as obligatory for the design of targeted DNA binders. The molecular docking studies were also performed for the newly synthesized hybrid structures and the results obtained strongly supported the theoretical calculations. Free energy values from dockings are found to be in good agreement with those of experimental values. The mechanism of interaction of these compounds from different conformers has found to be the intercalation. Furthermore, docking studies of the synthesized compounds confirmed both the qualitative as well as quantitative results.

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Inflammation is associated with numerous diseases like atherosclerosis, asthma, Alzheimer’s disease, gout, multiple sclerosis, osteoarthritis, rheumatoid arthritis, diabetes mellitus, carcinoma, bacterial or viral infections, etc. which result in chronic inflammation (1). Nonsteroidal anti-inflammatory drugs (NSAIDs) are the first choice of drugs for the treatment of various inflammatory diseases (2). They block prostaglandin synthesis by cyclooxygenases. The major side effect with chronic use of NSAIDs is gastrointestinal ulcerations due to inhibition of cyclooxygenase in tissues. The two COX isoforms, constitutively expressed COX-1 in most tissues, while COX-2 is induced at the site of inflammation (3), led to the development of selective COX-2 inhibitors that significantly reduce gastric ulceration associated with chronic use of NSAIDs. However, some selective COX-2 inhibitors are withdrawn from the market because of cardiovascular toxicity (4). So a real need exists to develop new anti-inflammatory and analgesic agents with enhanced efficacy, less toxicity and gastric ulceration.

Benzimidazole is an important pharmacophore possessing wide range of biological activities such as anti-allergic (5), antimicrobial (6) antioxidant (7), PARP (Poly ADP ribose polymerase) inhibitors- as anticancer (8), cytomegalovirus (HCMV) inhibitors (9), antilulcer (10), anti-inflammatory (11) and as antihistaminics (12). Substitution of benzimidazole at 1, 2, 5 and 6 positions accomplish the minimum structural requirements for anti-inflammatory and analgesic activity (13, 14) along with this benzimidazole nucleus substituted with an appropriate group at 2- position is an essential structural feature for gastric safety of the molecule (15). These characteristic features create benzimidazole an important therapeutic target for drug development.

Mannich bases are the end products of Mannich reaction and are known as beta amino ketone carrying compounds (16). In Mannich reaction, formaldehyde is condensed with primary or secondary amine and a compound containing active hydrogen. Introduction of a basic functional group rendering the molecule in aqueous solvent, makes Mannich base very reactive and are easily trans-
Scheme 1a. Synthesis of 2-substituted benzimidazole using o-phenylene diamine dihydrochloride

\[
\text{o-phenylene diamine dihydrochloride} \rightarrow \text{2-substituted benzimidazole}
\]

R

\[2a \quad \text{Cl}_2C_H_3\]

Scheme 1b. Synthesis of 2-substituted benzimidazoles using o-phenylene diamine

\[
\text{orthophenylene diamine} \rightarrow \text{Ar CHO} \rightarrow \text{Ar}
\]

Scheme 2. Synthesis of Mannich bases from 2-substituted benzimidazoles

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Compound</th>
<th>Ar</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>-CH_2Cl</td>
<td>3h</td>
<td>2-Br-C_H_3</td>
</tr>
<tr>
<td>3b</td>
<td>-CH_3</td>
<td>3i</td>
<td>3-Br-C_H_3</td>
</tr>
<tr>
<td>3c</td>
<td>3-CH_2N_2</td>
<td>3j</td>
<td>4-Br-C_H_3</td>
</tr>
<tr>
<td>3d</td>
<td>2-OH-C_H_2</td>
<td>3k</td>
<td>4-NO_2-C_H_3</td>
</tr>
<tr>
<td>3e</td>
<td>2-Cl-C_H_2</td>
<td>3l</td>
<td>4-F-C_H_3</td>
</tr>
<tr>
<td>3f</td>
<td>3-Cl-C_H_2</td>
<td>3m</td>
<td>2-NH_2-C_H_3</td>
</tr>
<tr>
<td>3g</td>
<td>4-Cl-C_H_2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Scheme 2. Synthesis of Mannich bases from 2-substituted benzimidazoles
formed into numerous other compounds (17). The reactivity of these bases accounts for their several pharmacological properties such as analgesic (18), anti-inflammatory (19, 20), anticancer (21, 22), anti-convulsant (23), antiviral, anthelmintic, antimalarial, antibacterial (24) antifungal (25, 26) and several other activities.

Looking into these findings together with diverse biological activities exhibited by benzimidazoles and less GI toxicity with 2-substituted benzimidazoles, also, increased activity by the formation of Mannich bases inspired us for the development of new Mannich bases of 2-substituted benzimidazole derivatives and evaluated for analgesic, anti-inflammatory and ulcerogenic activity. In silico method was used for the screening of novel compounds derived from mannich reaction. The target compounds after screening were further synthesized and the structures of all the compounds were confirmed by elemental and spectral analysis.

MATERIALS AND METHODS

Docking studies

Molecular docking studies were performed using Autodock Vina with pdb id: 1CX2. All the ligands employed in this study, were drawn using Marvin Sketch application of ChemAxon. The 3D structures of the receptor were obtained from Protein Data Bank (www.rcsb.org). Here, molecular docking simulations were performed for anti-inflammatory (PDB id: 1CX2) using Autodock tools of mgl lab for further pre-processing of our ligands and receptor proteins (27). In the pre-processing step, gasteiger charges were added both to the ligand as well as receptor. All the non-polar hydrogens were merged and water of crystallization was removed using the same graphical interface. Autodock tool uses the hybrid global-local search algorithm which is a big improvement on the genetic algorithm for the best confirmations of ligands. Autodock Vina requires 3 calculated grid maps for making our docking calculations extremely fast. The Autodock Vina uses a hybrid scoring function (empirical + knowledge based function) for evaluating the binding affinity of ligands with the receptor (28).

A set of 24 compounds was screened for anti-inflammatory activity by molecular docking simulations using pdb id: 1CX2. The screening resulted in 13 hit compounds which were further synthesized and evaluated for anti-inflammatory, analgesic and ulcerogenic activity.

Chemistry

Melting points were determined in open capillary tubes and are uncorrected. The purity of the synthesized compounds was checked on precoated aluminium silica gel G plates using UV/ iodine chamber as visualizing agent. Infrared spectra were recorded on Perkin Elmer Spectrum FTIR spectrophotometer using the potassium bromide disc method. Proton NMR spectra were recorded on Bruker Avance II (400 MHz) NMR spectrometer using DMSO-d$_6$ as a solvent and tetramethyl silane (TMS) as internal standard. Chemical shift values were expressed in delta parts per million (δ ppm). Elemental analysis was done using Eager Xperience CHN analyzer. Mass spectra were recorded on WATERS, Q-TOF MICROMASS spectrometer. 2-substituted benzimidazole Mannich bases were synthesized by the reaction of 2-substituted benzimidazole (secondary amine), formalin and benzamide (active hydrogen compound) (3a-m). 2-substituted benzimidazoles (2a-m) were synthesized by the reaction of ortho-phenylenediamine with substituted carboxylic acid (aliphatic moiety) and with substituted aromatic aldehyde (aromatic moiety), respectively. The compounds were characterized by spectral and analytical techniques.

General method

The title compounds were prepared by the following steps:

**Synthesis of 2-substituted benzimidazole from ortho-phenylenediamine dihydrochloride [2a]**

2-Substituted benzimidazole was synthesized by the reaction of o-phenylenediamine dihydrochloride with substituted carboxylic acid by the method described in the literature (29).

**Synthesis of 2-substituted benzimidazoles from ortho-phenylenediamine [2(b-m)]**

2-Substituted benzimidazoles were synthesized by the reaction of o-phenylenediamine and substituted benzaldehyde by the procedure reported in the literature (30).
Synthesis of Mannich base of 2-substituted benzimidazoles with benzamide [3(a-m)]

2-Substituted benzimidazole (0.01 M) was added to the ethanolic solution of benzamide (0.01 M). Formaldehyde (37-41% w/v) (0.01 M) was added and the reaction mixture was then adjusted to the pH of 3.5 with concentrated HCl. The mixture was kept at efficient ice cooling for half an hour. Then, it was refluxed with stirring at 80°C for 10-12 h. Formaldehyde (37-41% w/v) was added to the reaction mixture in portions for completion of the reaction. End of reaction was monitored by TLC. Reaction mixture was kept in refrigerator overnight. Solvent was evaporated under reduced pressure and product was collected, washed with water and recrystallized from ethanol.

By adopting the same procedures and taking equimolar quantities of reactants, 13 new derivatives were synthesized. Synthetic method for preparation of novel compounds [3(a-m)] is shown in general schemes.

Spectral data:

N-(2-chloromethyl-benzimidazol-1-yl methyl)-benzamide (3a)

Yellow crystals; m.p. 190-191°C, IR (KBr, cm⁻¹): 3308 (N-H), 1488 (C=N), 3056 C-H (CH₂), 2965 (C-H, Ar) 1526 (C=C), 1634 (C=O), 789 (C-Cl), 675-870 (CH bend. Ar). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.4-8.1 (m, 9H, ArH), 4.92 (s, 2H, NCH₂N), 9.0 (s, 1H, NH), 5.19 (s, 2H, CH₃). Analysis: calcd. for C₁₆H₁₄ClN₃O: C 64.11, H 4.71, N 14.02%; found: C 65.06, H 4.41, N 13.9%. MS: m/z = 300.2 (M+1).

N-(2-phenyl-benzimidazol-1-yl methyl)-benzamide (3b)

Brown crystals; m.p. 95-197°C, IR (KBr, cm⁻¹): 3308 (N-H), 1485 (C=NH), 3055 C-H (CH₃), 2966 (C-H, Ar) 1526 (C=C), 1634 (C=O), 789 (C-Cl), 675-870 (CH bend. Ar). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.4-8.1 (m, 9H, ArH), 4.92 (s, 2H, NCH₂N), 9.0 (s, 1H, NH), 5.19 (s, 2H, CH₃). Analysis: calcd. for C₁₆H₁₄ClN₃O: C 64.11, H 4.71, N 14.02%; found: C 65.06, H 4.41, N 13.9%. MS: m/z = 300.2 (M+1).

Table 2. Data of dock score and interactive amino acids.

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Design and synthesis of N-(benzimidazol-1-yl methyl)-benzamide... 1417

(C-H, Ar) 1526 (C=C), 1634 (C=O), 675-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.4-8.32 (m, 14H, ArH), 4.93 (s, 2H, NCH2N), 9.0 (s, 1H, NH). Analysis: calcd. for C21H17N3O: C 77.04, H 5.23, N 12.84%; found: C 76.3, H 5.20, N 12.72%. MS: m/z = 329.2 (M+1).

N-(2-pyridin-3-yl-benzimidazol-1-yl methyl)-benzamide (3c)

Yellow crystals; m.p. 205-206°C, IR (KBr, cm⁻¹): 3308 (N-H), 1486 (C=O), 1635 (C=O), 675-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.4-8.32 (m, 14H, ArH), 4.93 (s, 2H, NCH2N), 9.0 (s, 1H, NH). Analysis: calcd. for C20H16N4O: C 73.15, H 4.9, N 17.6%; found: C 72.06, H 5.6, N 16.92%. MS: m/z = 329.3 (M+1).

N-[2-(2-hydroxyphenyl)-benzimidazol-1-yl methyl]-benzamide (3d)

White crystals; m.p. 180-182°C, IR (KBr, cm⁻¹): 3310 (N-H), 1491 (C=O), 1633 (C=O), 675-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.0-8.18 (m, 13H, ArH), 4.91 (s, 2H, NCH2N), 9.0 (s, 1H, NH), 13.6 (br.s, 1H, OH). Analysis: calcd. for C21H17N3O2: C 73.45 , H 4.99 , N 12.24%; found: C 72.06, H 4.63, N 11.92%. MS: m/z = 344.8 (M+1).

N-[2-(2-chlorophenyl)-benzimidazol-1-yl methyl]-benzamide (3e)

White crystals; m.p. 190-192°C, IR (KBr, cm⁻¹): 3307 (N-H), 1485 (C=O), 1633 (C=O), 675-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.41-8.9 (m, 13H, ArH), 4.91 (s, 2H, NCH2N), 9.01 (s, 1H, NH). Analysis: calcd. for C21H16ClN3O: C 69.71, H 4.46, N 11.61%; found: C 68.5, H 4.31, N 10.2%. MS: m/z = 362.2 (M+1), 363(M+2).

N-[2-(3-chlorophenyl)-benzimidazol-1-yl methyl]-benzamide (3f)

White crystals; m.p. 200-202°C, IR (KBr, cm⁻¹): 3309 (N-H), 1486 (C=O), 1633 (C=O), 675-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.46-8.7 (m, 13H, ArH), 4.98 (s, 2H, NCH2N), 9.0 (s, 1H, NH). Analysis: calcd. for C21H16ClN3O: C 69.71, H 4.46, N 11.6%; found: C 68.5, H 4.81, N 11.2%.

N-[2-(4-chlorophenyl)-benzimidazol-1-yl methyl]-benzamide (3g)

White crystals; m.p. 195-196°C, IR (KBr, cm⁻¹): 3307 (N-H), 1486 (C=O), 1635 (C=O), 765-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.35-8.6 (m, 13H, ArH), 4.96 (s, 2H, NCH2N), 8.90 (s, 1H, NH). Analysis: calcd. for C21H16ClN3O: C 69.71, H 4.46, N 11.61%; found: C 68.52, H 4.31, N 10.8%. MS: m/z = 363.9 (M+2).

N-[2-(2-bromophenyl)-benzimidazol-1-yl methyl]-benzamide (3h)

White crystals; m.p. 190-192°C, IR (KBr, cm⁻¹): 3309 (N-H), 1488 (C=O), 1634 (C=O), 765-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.1-8.1 (m, 13H, ArH), 4.91 (s, 2H, NCH2N), 8.9 (s, 1H, NH). Analysis: calcd. for C21H16BrN3O: C 62.08, H 3.97, N 10.34%; found: C 63.3, H 2.98, N 9.43%. MS: m/z = 406.2 (M+1), 407.3 (M+2).

N-[2-(3-bromophenyl)-benzimidazol-1-yl methyl]-benzamide (3i)

White crystals; m.p. 192-194°C, IR (KBr, cm⁻¹): 3307 (N-H), 1487 (C=O), 1634 (C=O), 765-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.1-8.7 (m, 13H, ArH), 4.98(s, 2H, NCH2N), 9.0 (s, 1H, NH). Analysis: calcd. for C21H16BrN3O: C 62.08, H 3.97, N 10.34%; found: C 61.3, H 2.96, N 9.49%.

N-[2-(4-bromophenyl)-benzimidazol-1-yl methyl]-benzamide (3j)

Off white crystals; m.p. 195-196°C, IR (KBr, cm⁻¹): 3308 (N-H), 1486 (C=O), 1633 (C=O), 675-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.2-8.6 (m, 13H, ArH), 4.97 (s, 2H, NCH2N), 8.89 (s, 1H, NH). Analysis: calcd. for C21H16BrN3O: C 61.9, H 2.96, N 9.82%.

N-[2-(4-nitrophenyl)-benzimidazol-1-yl methyl]-benzamide (3k)

Yellow crystals; m.p. 180-183°C, IR (KBr, cm⁻¹): 3307 (N-H), 1485 (C=O), 1634 (C=O), 675-870 (CH bend. Ar). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 7.24-8.46 (m, 13H, ArH), 4.94 (s, 2H, NCH2N), 8.90 (s, 1H, NH). Analysis: calcd. for C21H16N4O3: C 67.73, H 4.46, N 11.6%; found: C 66.3, H 4.28, N 14.96%. MS: m/z = 373.7 (M+1).

N-[2-(4-chlorophenyl)-benzimidazol-1-yl methyl]-benzamide (3l)
White crystals; m.p. 230-231°C, IR (KBr, cm⁻¹): 3310 (N-H), 1458 (C=N), 3055 C-H (CH₂), 2927 (C-H, Ar) 1525 (C=C), 1634 (C=O), 848 (C-F), 675-870 (CH bend. Ar). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 7.4-8.48 (m, 13H, ArH), 4.90 (s, 2H, NCH₂N), 9.03 (s, 1H, NH). Analysis: calcd. for C₂₁H₁₆FN₃O: C 73.03, H 4.67, N 12.17%; found: C 72.7, H 3.98, N 11.82%. MS: m/z = 346.3 (M+1).

Yellow crystals; m.p. 206-208°C, IR (KBr, cm⁻¹): 3310 (N-H), 1486 (C=N), 3086 C-H (CH₂), 2959 (C-H, Ar) 1525 (C=C), 1634 (C=O), 675-870 (CH bend. Ar). ¹H NMR (400 MHz, DMSO-d₆, δ, ppm): 6.52-7.95 (m, 13H, ArH), 4.98 (s, 2H, NCH₂N), 8.79 (s, 1H, NH), 4.0 (s, 2H, NH₂). Analysis: calcd. for

Table 3. Physical data of synthesized compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Melting point (OC)</th>
<th>Yield (%)</th>
<th>Rf value</th>
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<td>C, H₂⁻</td>
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<td>195-197</td>
<td>80.4</td>
<td>0.95</td>
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<td>190-191</td>
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<td>0.87</td>
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<td>190-192</td>
<td>75.4</td>
<td>0.81</td>
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<td>192-194</td>
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<td>0.80</td>
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<td>195-196</td>
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Mobile phase -CHCl₃ : CH₃OH: 9.5: 0.5.
Design and synthesis of N-(benzimidazol-1-yl methyl)-benzamide... 1419

Figure 2. Binding pose for internal ligand SC-558 (dock score -7.6 kcal/M) within the domain of COX-2 receptor showing hydrogen bonding in dashed green line

Table 4. Acute toxicity data of synthesized compounds.

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<td>3b</td>
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<tr>
<td>3c</td>
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<td>3d</td>
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<td>0</td>
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<td>3e</td>
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<td>0</td>
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<td>3f</td>
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<tr>
<td>3m</td>
<td>(1g/kg)</td>
<td>6</td>
<td>0</td>
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**Pharmacological studies**

*Animals*

Swiss albino mice (20-25 g) and albino Wistar rats (150-200 g) of either sex were used for current animal study. All the animals were housed in groups of 6 per cage at a temperature of 25 ± 1°C and a relative humidity of 45-55%. A 12 h dark and 12 h light cycle was followed during the experiments. Animals were allowed free access to food and water *ad libitum*. All the animal experiments were performed with the approval of the Institutional Animal Ethics Committee, Chitkara College of Pharmacy, Punjab, India (Regd. No. 1181/PO/Ebi/08/CPCSEA).

**Acute toxicity studies**

The acute toxicity studies were performed in groups of six Swiss albino mice, weighing 25 ± 2 g which were fasted overnight and treated orally with test compounds with a dose of 1000 mg/kg body weight in evaluating their toxic effect as per OECD.

**Table 5. Anti-inflammatory activity of tested compounds and diclofenac sodium.**

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<th>No.</th>
<th>Treatments</th>
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<th>Percentage (%) edema at 4 h</th>
<th>Percentage (%) reduction in edema</th>
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<td>Vehicle control (DMSO) (1mg/mL)</td>
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<tr>
<td>2.</td>
<td>Diclofenac sodium (positive control)</td>
<td>(50 mg/kg)</td>
<td>21.75 ± 1.25</td>
<td>76.25 ± 3.75</td>
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<td>3.</td>
<td>3a (100 mg/kg)</td>
<td>29.50 ± 1.55</td>
<td>70.50 ± 2.25</td>
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<tr>
<td>4.</td>
<td>3b (100 mg/kg)</td>
<td>73.85 ± 3.55</td>
<td>26.15 ± 1.20</td>
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<tr>
<td>5.</td>
<td>3c (100 mg/kg)</td>
<td>68.35 ± 3.05</td>
<td>31.65 ± 1.29</td>
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<td>6.</td>
<td>3d (100 mg/kg)</td>
<td>71.25 ± 3.25</td>
<td>28.75 ± 1.34</td>
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<td>7.</td>
<td>3e (100 mg/kg)</td>
<td>30.34 ± 1.25</td>
<td>69.66 ± 2.75</td>
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<td>8.</td>
<td>3f (100 mg/kg)</td>
<td>65.28 ± 3.75</td>
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<tr>
<td>9.</td>
<td>3g (100 mg/kg)</td>
<td>34.18 ± 1.5</td>
<td>65.82 ± 2.15</td>
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<td>10.</td>
<td>3h (100 mg/kg)</td>
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<td>66.19 ± 2.25</td>
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<tr>
<td>11.</td>
<td>3 (100 mg/kg)</td>
<td>60.23 ± 2.78</td>
<td>39.77 ± 1.46</td>
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<tr>
<td>12.</td>
<td>3j (100 mg/kg)</td>
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<td>63.41 ± 2.10</td>
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<td>13.</td>
<td>3l (100 mg/kg)</td>
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<td>49.75 ± 1.95</td>
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<td>14.</td>
<td>3m (100 mg/kg)</td>
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<td>44.75 ± 1.5</td>
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</tbody>
</table>

Values are the mean ± standard error of mean (SEM) (n = 6). *Statistically significant compared to control group (p ≤ 0.05). All the statistical analyses were performed by one-way ANOVA followed by Tukey’s test as a post hoc analysis.
For anti-inflammatory activity, Wistar rats of either sex were divided into vehicle control, standard (positive control) and different test groups of six rats each. All test compounds, vehicle control and reference drug were given orally to the respective groups as a suspension in dimethyl sulfoxide (10% DMSO in water, 10 mL/kg), one hour before carrageenan injection (31). The procedure followed was, acute inflammation produced by injection of carrageenan (0.1 mL of 1% w/v suspension) (32), in the right hind paw of the rats under the plantar aponeurosis. It was injected +1 h after the oral administration of the drug. The paw edema volume was calculated by plethysmograph using mercury displacement method at 0 h and 4 h (immediately after injection and 4 h post injection of carrageenan). The percent anti-inflammatory activity was calculated according to the formula given below:

\[
\text{Percentage inhibition of paw edema} = \left(1 - \frac{V_t}{V_c}\right) \times 100
\]

where \(V_c\) represents an average increase in paw volume (average inflammation) of the control group of rats at a given time; and \(V_t\) was the average inflammation of the test compound treated rats at the same time.

(Organization for Economic Co-operation and Development) 423 guidelines.

**Anti-inflammatory activity by carrageenan-induced rat paw edema method**

For anti-inflammatory activity, Wistar rats of either sex were divided into vehicle control, standard (positive control) and different test groups of six rats each. All test compounds, vehicle control and reference drug were given orally to the respective groups as a suspension in dimethyl sulfoxide (10% DMSO in water, 10 mL/kg), one hour before carrageenan injection (31). The procedure followed was, acute inflammation produced by injection of carrageenan (0.1 mL of 1% w/v suspension) (32), in the right hind paw of the rats under the plantar aponeurosis. It was injected +1 h after the oral administration of the drug. The paw edema volume was calculated by plethysmograph using mercury displacement method at 0 h and 4 h (immediately after injection and 4 h post injection of carrageenan). The percent anti-inflammatory activity was calculated according to the formula given below:

\[
\text{Percentage inhibition of paw edema} = \left(1 - \frac{V_t}{V_c}\right) \times 100
\]

where \(V_c\) represents an average increase in paw volume (average inflammation) of the control group of rats at a given time; and \(V_t\) was the average inflammation of the test compound treated rats at the same time.
Acetic acid-induced writhing assay

Acetic acid-induced writhing test was used to evaluate the analgesic potential of most potent compounds showing anti-inflammatory activity (33). Swiss albino mice were divided into vehicle control, standard (positive control) and different test groups of six mice each. The vehicle control group was administered p.o., with dimethyl sulfoxide (10% DMSO in water, 10 mL/kg), whereas positive control [indomethacin (30 mg/kg)] and test compounds were administered at a dose level of 100 mg/kg suspended in 10% DMSO p.o., 30 min before the i.p. injection of the acetic acid solution at a dose of 1 mL/kg. The number of wriths per animal was recorded for 15 min. The analgesic activity was calculated using the following formula and expressed as percentage protection and the results are presented in Table 6 and Fig. 4.

\[
\% \text{ Analgesic activity} = \frac{\text{No. of writhings of control} - \text{No. of writhings of test compound}}{\text{No. of writhings of control}} \times 100
\]

Final effective dose of compounds was selected by performing pilot studies.

Ulcerogenic assay

Wistar rats of either sex weighing 150-200 g were divided into respective groups like vehicle control [2% sodium carboxymethyl cellulose (CMC)], reference control (indomethacin) and different test compound groups (n = 6). The test compounds and reference drug were administered to animals orally at a dose of 100 mg/kg and 30 mg/kg, respectively, by dissolving in 2% sodium carboxymethyl cellulose (CMC) as a vehicle. After 6 h, the rats were sacrificed for ulcerogenic activity, and their stomach was removed. Formalin (10% v/v) was then injected into the suture ligated stomach for further examination. The stomach was opened, washed in normal saline, and examined under magnifier for measuring the length of lesions. The induced ulcers appeared as small spots punctiform lesions and each was given a score between 1 and 4. Ulcers of 0.5 mm diameter were assigned a score of 1 whereas ulcers of diameters 1 and 2 mm were given scores of 2 and 4, respectively. Stomach with no lesions was assigned a score of zero. Summed to give a total lesion score (in mm) for each animal, the mean count for each group being calculated (34) and results are depicted in Table 7.

Statistical analysis

All the results were expressed as the mean ± standard error of mean (SEM). Data of the results were analyzed by using one-way variance (ANOVA) followed by Tukey’s test. A value of p < 0.05 was considered as statistically significant. The SIGMA Plot 13, Systat Software, Inc. 2107 North First Street, Suite 360, San Jose, CA 95131 USA, was used for statistical analysis.

RESULTS AND DISCUSSION

Docking study

Before in vivo evaluation, it was thought worthy to study the interaction of compounds to be synthesized with COX-2 receptor using molecular docking studies. The purpose of the study was to screen the compounds in silico for further in vivo evaluation. Considering 1CX2 as target, the series of compounds were docked to get the best in silico confirmations in the domain of selective COX-2 inhibitor.

In the present study, H-bonding and dock score were kept in consideration to get the hits among the set of compounds for the analysis. From the in silico study, 13 hit compounds with dock score higher than that of internal ligand SC-558 (-7.6 kcal/M), were further synthesized and

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Lesion score (mm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Vehicle control (2% CMC)</td>
<td>1.0 ± 0.25^c</td>
</tr>
<tr>
<td>2.</td>
<td>Indomethacin (reference control)</td>
<td>32.50 ± 2.50</td>
</tr>
<tr>
<td>3.</td>
<td>3a</td>
<td>13.90 ± 0.95^c</td>
</tr>
<tr>
<td>4.</td>
<td>3e</td>
<td>15.85 ± 1.18^c</td>
</tr>
<tr>
<td>5.</td>
<td>3g</td>
<td>18.90 ± 1.85</td>
</tr>
<tr>
<td>6.</td>
<td>3h</td>
<td>16.55 ± 1.25^c</td>
</tr>
<tr>
<td>7.</td>
<td>3j</td>
<td>20.45 ± 2.05</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM (n = 6). ^cStatistically significant compared to indomethacin (p ≤ 0.05). All the statistical analysis was performed by one-way ANOVA followed by Tukey’s test as post hoc analysis.
evaluated for anti-inflammatory, analgesic and ulcerogenic activity. The main amino acids which played a vital role in interaction are Lys194, Asn292, Arg255, Gly99 and Glu193. Among the series, compound 3a has displayed best dock score of -9.0 kcal/mol with 2 H-bonds, length of 2.216 Å and 2.036 Å, respectively, shown in Figure 1. Similarly, compound 3e showed dock score of -8.9 kcal/M with 1 hydrogen bond and main amino acid which played interaction is Glu193. The parameters of grid box are given in Table 1. The data of dock score and interactive amino acids of series are provided in Table 2. The secondary structure of the target receptor is shown in Figure 2. From the docking study, we predicted that the compounds showing good dock score as well as interaction with the receptor would possess better activity.

Chemistry
In this study, on the basis of docking studies out of 24 derivatives, 13 novel compounds incorporating the scaffold of benzimidazole were synthesized and screened for anti-inflammatory, analgesic and ulcerogenic activity. Synthesis of the compounds was carried out as outlined in general schemes. Benzamide (active hydrogen compound) was reacted with secondary amine (2-substituted benzimidazole) in the presence of formalin and conc. hydrochloric acid to furnish the Mannich bases. These were characterized on the basis of their elemental and spectral analysis. From IR spectra, the appearance of peaks at 3308-3310 cm⁻¹ and 1400-1500 cm⁻¹ indicated the presence of NH of carboxamide and C=N stretching of benzimidazole, respectively. From NMR spectra, a sharp singlet at 4.8-4.99 ppm ascertained the presence of CH₂ attached to NH in all the synthesized compounds. The appearance of singlet at 8.0-8.9 ppm confirmed the presence of NH (D₂O exchangeable) of carboxamide in all the synthesized compounds. The appearance of multiplet at 6.30-8.0 ppm indicated the presence of aromatic and hetero-aromatic protons. In addition, compound 3a showed singlet at 5.19 ppm for 2H of CH₂. The appearance of broad singlet at 13.6 ppm confirmed the presence of one proton of hydroxyl functionality in compound 3d. The calculated molecular weight of the synthesized compounds was matched with an observed m/e value. Hence, data obtained were found to be in good firmity with the calculated values of the proposed structure. Physical data of synthesized compounds are given in Table 3.

Animal studies
Acute toxicity of test compounds
Out of 13 compounds, only one compound 3k showed toxic effect and mortality in mice at a dose of 1g/kg p.o., out of six animals in 3k treatment group, 3 animals were dead after treatment. Rest all compounds with a dose of 1g/kg, were nontoxic as they have not shown mortality and adverse effects (lethargy and convulsions) in any group (12 groups). Acute toxicity data of synthesized compounds are given in Table 4.

Anti-inflammatory activity of test compounds
From Table 5, it was found that out of 12 compounds, five compounds showed significant results in comparison with standard diclofenac sodium as positive control. Amongst all the title compounds, 3a, 3e, 3g, 3h and 3j showed significant (p < 0.05) potent anti-inflammatory activity as compared to vehicle control group (10% DMSO) and rest of the compounds showed moderate or low activity.

Analgesic activity of test compounds
On the basis of anti-inflammatory activity only five compounds were selected for analgesic activity. Fig. 4 and Table 6 revealed that all the compounds 3a, 3e, 3g, 3h and 3j showed significant (p < 0.05) potent analgesic and percentage protection analgesic activity of test compounds, respectively, as compared to vehicle control group (10% DMSO).

Ulcerogenic activity
On the basis of efficacy and potency of anti-inflammatory and analgesic activity, five compounds were selected for ulcerogenic studies. These compounds also showed significant (p < 0.05) results for ulcerogenic activity as compared to indomethacin as reference drug. Table 7 revealed that compound 3a, 3e and 3h were most potent among five compounds used for respective study.

CONCLUSION
A series of N-(benzimidazol-1-yl methyl)-benzamide derivatives were initially screened by docking simulations using COX-2 receptor (PDB ID: 1CX2). Binding affinities of the synthesized compounds were evaluated by using docking program Autodock Vina. The main amino acids that played a vital role in interaction with COX-2 receptor are Lys194, Asn292, Arg255,
Gly99 and Glu193. The designed derivatives having dock score higher than internal ligand, were synthesized with remarkable yields and their structures were elucidated by spectral analysis. Selected compounds were screened for their in vivo anti-inflammatory and analgesic activity and five out of twelve compounds showed significant results as compared to disease control groups in animal studies. Based on anti-inflammatory and analgesic activity, compounds 3a (dock score -9.0 kcal/M), 3e (dock score -8.9 kcal/M), 3g (dock score -8.7 kcal/M) and 3j (dock score -8.4 kcal/M) were selected for ulcerogenic activity. The compounds 3a, 3e and 3h were found to be safe on gastric mucosa as indicated by their low lesion score 13.90 ± 0.95, 15.85 ± 1.18 and 16.55 ± 1.25, respectively, as compared to indomethacin (32.50 ± 2.50). Structure activity relationship revealed that compounds substituted with electron withdrawing groups (Cl, Br) at ortho position of phenyl ring and chloromethyl group at 2-position in benzimidazole nucleus were found to be most active anti-inflammatory, analgesic and non-ulcerogenic agents. The pharmacological activities exhibited by newly synthesized compounds have confirmed their novelty as safer therapeutic agents. Both the hypothesis (docking) and practice (anti-inflammatory, analgesic and ulcerogenic activity) reveal that N-(benzimidazol-1-yl methyl)-benzamide derivatives act as potent inhibitor of 1CX2 receptor. Hence it is concluded that N-(benzimidazol-1-yl methyl)-benzamide derivatives with electron withdrawing substituents could be used for designing more potent anti-inflammatory and analgesic agents with less gastric lesions. Further studies of these derivatives are highly recommended to assess the safety of novel compounds.

Acknowledgments

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Conflict of interests

The authors declare that there is no conflict of interests.

REFERENCES


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Cancer still remains a mean threat to human health; it is a major cause of death worldwide. Therefore, the development of highly efficient, selective and less noxious antitumor drug remains an urgent need (1).

The design and synthesis of new pyrazole derivatives has been a unique position in the drug discovery due to their broad range of biological activities, such as anti-inflammatory, antiangiogenic, antibacterial, antimicrobial, anticancer, antioxidant, anti-influenza and analgetic activities (2-9). Moreover, pyrazole unit are important core structures in a number of natural products (10-13). Pyrazole is versatile lead compound for designing potent anticancer compounds. Several recent studies suggest pyrazole derivatives as promising anticancer agents, indicating their use in the development of new anticancer agents (14-18). The important role of pyrazoles in antitumor agents due to their good inhibitory activity against BRAF(V600E), EGFR, telomerase, ROS Receptor Tyrosine Kinase and Aurora-A kinase (19). In addition, N-substituted pyrazoles have been implemented as anti-leukemic, antitumor, anti-proliferative, anti-angiogenic, DNA interacting, proapoptotic, autophagy, and anti-tubulin agents. These compounds exhibited remarkable anti-cancer effects through inhibition of different types of enzymes, proteins and receptors which play critical role in cell division (20).

On the other hand, we expected that compound 4 and 5 are interesting pyrazole derivatives with possible anticancer activity. In continuation to our work effort to develop new anticancer agents (21), we reported herein a series of novel functionalized pyrazole derivatives starting from compounds (4) or (5) to evaluate their in vitro anti-tumor activities against HepG-2, PC-3 and HCT-116 human carcinoma cell lines using MTT assay. Eight compounds showed good anticancer activities against HCT-116 carcinoma cells and five compounds showed good anticancer activities against PC-3 cancer cells but all the compounds showed weak or no anticancer activities against HepG-2 liver cancer.

EXPERIMENTAL

Chemistry

General

All melting points were uncorrected and measured using an Electro-thermal IA 9100 apparatus (Shimadzu, Japan). Microanalytical data were performed by Vario El-Mentar apparatus (Shimadzu, Japan), National Research Centre, Cairo, Egypt. IR spectra were recorded (as potassium bromide pellets) using KBr disc technique on a Perkin-Elmer 1650 Spectrophotometer, National Research Centre,
Cairo, Egypt. NMR experiments were determined on a JEOL-Ex-300 MHz in deuterated dimethyl sulfoxide (DMSO-d$_6$) and chemical shifts were expressed as parts per million; ppm (δ values) against TMS as an internal reference (Faculty of Science, Cairo University, Cairo, Egypt). Mass spectra were recorded on Shimadzu GCMS-QP-1000EX mass spectrometer at 70 eV (Cairo University, Cairo, Egypt).

**Ethyl 3-(4-methoxyphenyl)-3-oxopropanoate (3)**

This compound was prepared according to the reported method (22) by the reaction of 4-methoxy acetophenone (1) and diethyl carbonate (2) in the presence of sodium hydride. Viscous oil, yield 93%; m.p. 167°C; IR (KBr, cm$^{-1}$): 3411-3194 (NH+OH), 1670 (C=O); 1H NMR (DMSO-d$_6$), δ ppm): 3.78 (s, 3H, -OCH$_3$), 3.81 (s, 2H, COCH$_2$CO), 6.87 (d, J = 9 Hz, 2H), 7.10 (d, J = 9 Hz, 2H, -CH=), 7.20 (d, J = 9 Hz, 2H, ArH), 9.71 (s, 1H, D$_2$O exchangeable, NH); MS (C$_8$H$_7$N$_2$O$_3$) m/z = 191 (M$^+$).

**5-(4-Methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (4)**

This compound was prepared according to the reported method (23) by the reaction of ethyl 3-(4-methoxyphenyl)-3-oxopropanoate (3) and phenylhydrazine refluxing in acetic acid for 10-15 min. Yield (94%); m.p. 220°C; IR (KBr, cm$^{-1}$): 3300-2600 (NH), 1670 (C=O); 1H NMR (DMSO-d$_6$), δ ppm): 3.79 (s, 3H, -OCH$_3$), 4.90 (s,1H, OH), 6.52-7.38 (m, 6H, ArH), 7.90 (d, J = 9 Hz, 2H, ArH), 10.03 (s, 1H, D$_2$O exchangeable, NH); MS (C$_{10}$H$_{10}$N$_2$O$_2$) m/z = 279 (M$^+$).

**5-(4-Methoxyphenyl)-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (5)**

This compound was prepared according to the reported method (23, 24) by the reaction of 4-methoxy phenyl-1-oxo-1,2,3,4-tetrahydro-6H-pyrazine (6a) and diethyl carbonate (2) in the presence of sodium hydride. Viscous oil, yield 93%; m.p. 145°C; IR (KBr, cm$^{-1}$): 3400-2600 (NH), 1670 (C=O); 1H NMR (DMSO-d$_6$), δ ppm): 3.79 (s, 3H, -OCH$_3$), 4.90 (s,1H, OH), 6.52-7.38 (m, 6H, ArH), 7.57 (d, J = 9 Hz, 2H, ArH), 9.71 (s, 1H, D$_2$O exchangeable, NH); MS (C$_{10}$H$_{10}$N$_2$O$_2$) m/z = 279 (M$^+$).

**Synthesis of 5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-ones (6a-j)**

To a mixture of 3-(4-methoxyphenyl)-1-pyrazol-5(4H)-one (4) or 3-(4-methoxyphenyl)-1-phenyl-1H-pyrazol-5(4H)-one (5) (1 mM) and the appropriate aldehyde (1 mM) in absolute ethanol (25 mL), few drops of piperidine were added. The reaction mixture was refluxed for 4 h, and then cooled to room temperature. The precipitate was filtered, dried and washed with ethanol. The crude product was recrystallized from EtOH/DMF to afford compounds 6a-j, respectively.

**4-Benzylidene-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (6a)**

Yield (70%); m.p. 110°C; IR (KBr, cm$^{-1}$): 3220 (NH), 1720 (C=O); 1H NMR (DMSO-d$_6$, δ ppm): 3.78 (s, 3H, -OCH$_3$), 6.60 (d, J = 9 Hz, 2H, ArH), 6.94-7.89 (m, 6H, 4ArH + -CH=), 7.90 (d, J = 8 Hz, 2H, ArH), 10.03 (s, 1H, D$_2$O exchangeable, NH); MS (C$_{17}$H$_{14}$N$_2$O$_2$) m/z = 279 (M$^+$).

**4-(4-Bromobenzylidene)-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (6b)**

Yield (80%); m.p. 140°C; IR (KBr, cm$^{-1}$): 3225 (NH), 1715 (C=O); 1H NMR (DMSO-d$_6$, δ ppm): 3.77 (s, 3H, -OCH$_3$), 6.95 (d, J = 9 Hz, 2H, ArH of 4-MeO-C$_6$H$_4$), 7.39 (s, 1H, -CH=), 7.60 (d, J = 9 Hz, 2H, ArH of 4-BrC$_6$H$_4$), 7.73 (d, J = 8 Hz, 2H, ArH of 4-BrC$_6$H$_4$), 7.88 (d, J = 8 Hz, 2H, ArH of 4-BrC$_6$H$_4$), 9.98 (s, 1H, D$_2$O exchangeable, NH); MS (C$_{17}$H$_{13}$BrN$_2$O$_2$) m/z = 358 (M$^+$).

**4-(Dimethylamino) benzylidene)-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (6c)**

Yield (79%); m.p. 130°C; IR (KBr, cm$^{-1}$): 3230 (NH), 1670 (C=O); 1H NMR (DMSO-d$_6$, δ ppm): 3.04 (s, 6H, 2CH$_3$), 3.78 (s, 3H, -OCH$_3$), 6.71 (d, J = 8.5 Hz, 2H, ArH of 4-(CH$_3$)$_2$N), 7.10 (d, J = 2.5 Hz, 2H, ArH), 7.38 (s, 1H, -CH=), 7.60 (d, J = 9 Hz, 2H, ArH), 7.69 (d, J = 9 Hz, 2H, ArH), 9.67 (s, 1H, D$_2$O exchangeable, NH); MS (C$_{18}$H$_{16}$N$_2$O$_4$) m/z = 322 (M$^+$).

**4-(4-Chlorobenzylidene)-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (6d)**

Yield (65%); m.p. 140°C; IR (KBr, cm$^{-1}$): 3220 (NH), 1680 (C=O); 1H NMR (DMSO-d$_6$, δ ppm): 3.77 (s, 3H, OCH$_3$), 6.58-8.0 (m, 8H, 7ArH+-CH=), 10.20 (s, 1H, D$_2$O exchangeable, NH); MS (C$_{17}$H$_{13}$BrN$_2$O$_2$) m/z = 348 (M$^+$).

**4-(4-Hydroxy-3-methoxybenzylidene)-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (6e)**

Yield (71%); m.p. 145°C; IR (KBr, cm$^{-1}$): 3411-3194 (NH+OH), 1670 (C=O); 1H NMR (DMSO-d$_6$, δ ppm): 3.78 (s, 3H, -OCH$_3$), 4.90 (s,1H, OH), 6.52-7.38 (m, 6H, 5ArH+-CH=), 7.57 (d, J = 9 Hz, 2H, ArH ), 9.71 (s, 1H, D$_2$O exchangeable, NH), MS (C$_{17}$H$_{15}$O$_3$N$_2$) m/z = 325 (M$^+$).

**4-(4-Hydroxy-3-methoxybenzylidene)-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (6f)**

Yield (71%); m.p. 145°C; IR (KBr, cm$^{-1}$): 3411-3194 (NH+OH), 1670 (C=O); 1H NMR (DMSO-d$_6$, δ ppm): 3.78 (s, 3H, -OCH$_3$), 3.81 (s, 2H, -OH), 6.52-7.38 (m, 6H, 5ArH+-CH=), 7.57 (d, J = 9 Hz, 2H, ArH ), 9.71 (s, 1H, D$_2$O exchangeable, NH), MS (C$_{17}$H$_{15}$O$_3$N$_2$) m/z = 325 (M$^+$).
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4-(3,4-Dimethoxybenzylidene)-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (6f)

Yield (66%); m.p. 170°C; IR (KBr, cm⁻¹): 3220 (NH), 1665 (C=O); δH NMR (DMSO-d₆, δ ppm): 3.77 (s, 3H, -OCH₃), 3.83 (s, 3H, -OCH₃), 6.83 (d, J = 9 Hz, 1H, vaniline), 6.97 (d, J = 9 Hz, 2H, MeOArH), 7.04 (m, 2H, Vaniline), 7.20 (t, J = 9 Hz, 1H, ArH), 7.40 (t, J = 9 Hz, 2H, ArH), 7.50 (s, 1H, -CH=), 7.52 (d, J = 9 Hz, 2H, MeOArH), 7.78 (d, J = 9 Hz, 2H, ArH), MS (C₁₉H₁₅N₃O₃) m/z = 339 (M⁺).

4-Benzylidene-5-(4-methoxyphenyl)-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (6g)

Yield (70%); m.p. 192°C; IR (KBr, cm⁻¹): 3180 (2NH), 1670 (C=O); δH NMR (DMSO-d₆, δ ppm): 2.32 (s, 3H, CH₃), 3.81 (s, 3H, -OCH₃), 7.05 (d, J = 9 Hz, 2H, ArH), 7.26 (d, J = 9 Hz, 2H, ArH), 7.48 (d, J = 9 Hz, 2H, ArH), 7.99 (d, J = 9 Hz, 2H, ArH), 11.94 (s, 1H, D₂O exchangeable, NH); MS (C₂₃H₁₈N₂O₂) m/z = 355 (M⁺).

4-(4-Bromobenzylidene)-5-(4-methoxyphenyl)-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (6h)

Yield (78%); m.p. 175°C; IR (KBr, cm⁻¹): 3300(2NH), 1670 (C=O); δH NMR (DMSO-d₆, δ ppm): 3.77 (s, 3H, -OCH₃), 6.94 (d, J = 9 Hz, 2H, ArH), 7.0 (d, J = 9 Hz, 2H, ArH), 7.53 (d, J = 9 Hz, 2H, ArH), 8.01 (d, J = 9 Hz, 2H, ArH), 11.72 (s, 1H, D₂O exchangeable, NH); MS (C₂₃H₁₈N₂O₂) m/z = 3244 (M⁺).

4-(4-Methoxyphenyl)-4-[2-(p-tolyl)hydrazineylidene]-2,4-dihydro-3H-pyrazol-3-one (6i)

Yield (78%); m.p. 213°C; IR (KBr, cm⁻¹): 3420 (2NH), 1660 (C=O); δH NMR (DMSO-d₆, δ ppm): 3.81 (s, 3H, -OCH₃), 7.04-7.33 (m, 2H, ArH), 7.57-7.64 (m, 2H, ArH), 8.01 (d, J = 9 Hz, 2H, ArH), 8.58 (d, J = 9 Hz, 2H, ArH), MS (C₂₃H₁₈N₂O₂) m/z = 398 (M⁺).

4-[4-Dimethylamino]benzylidene]-5-[4-methoxyphenyl]-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (6j)

Yield (60%); m.p. 180°C; IR (KBr, cm⁻¹): 3150 (OH), 1670 (C=O); δH NMR (DMSO-d₆, δ ppm): 3.77 (s, 3H, -OCH₃), 3.83 (s, 3H, -OCH₃), 6.83 (d, J = 9 Hz, 1H, vaniline), 6.97 (d, J = 9 Hz, 2H, MeOArH), 7.04 (m, 2H, Vaniline), 7.20 (t, J = 9 Hz, 1H, ArH), 7.40 (t, J = 9 Hz, 2H, ArH), 7.50 (s, 1H, -CH=), 7.78 (d, J = 9 Hz, 2H, MeOArH), 7.99 (d, J = 9 Hz, 2H, ArH), MS (C₁₉H₁₉FN₂O₂) m/z = 401 (M⁺).
7.46 (d, J = 9 Hz, 2H, ArH), 7.57 (d, J = 9 Hz, 2H, ArH), 8.01 (d, J = 9 Hz, 2H, ArH), 11.90 (s, 1H, D₂O exchangeable, NH), 13.81 (s, 1H, D₂O exchangeable, NH); MS (C₁₆H₁₃ClN₄O₂) m/z = 389 (M⁺).

4-[2-(4-Chlorophenyl)hydrazineylidene]-5-(4-methoxyphenyl)-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (7j)

Yield (80%); m.p. 195°C; IR (KBr, cm⁻¹): 3310 (NH), 1660 (C=O); ¹H NMR (DMSO-d₆, δ, ppm): 3.82 (s, 3H, -OCH₃), 7.06 (d, J = 9 Hz, 2H, MeOArH), 7.24 (t, J = 9Hz, 2H, ArH), 7.45 (t, J = 9 Hz, 2H, ArH), 7.64 (d, J = 9 Hz, 2H, ArH), 7.90 (d, J = 12 Hz, 2H, ArH), 8.00 (d, J = 12 Hz, 2H, ArH), 8.13 (d, J = 12 Hz, 2H, ArH), 13.60 (s, 1H, D₂O exchangeable, NH); MS (C₂₂H₁₇ClN₄O₂) m/z = 405 (M⁺).

**Synthesis of 3-[3-(4-methoxyphenyl)-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene] indolin-2-one derivatives (9a-d)**

To a mixture of pyrazole 4 or 5 (1 mM) and isatin (1 mM) in glacial acetic acid (25 mL), 3 mM of anhydrous sodium acetate were added. The reaction mixture was refluxed for 4 h, and then cooled to room temperature. The precipitate was filtered, dried and washed with ethanol. The crude product was recrystallized from EtOH/DMF to afford compounds 9a-d, respectively.

3-[3-(4-Methoxyphenyl)-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene] indolin-2-one (9a)

Yield (65%); m.p. >300°C; IR (KBr, cm⁻¹): 3250 (2NH), 1700-1660 (2C=O); ¹H NMR (DMSO-d₆, δ, ppm): 3.77 (s, 3H, -OCH₃), 6.70-7.95 (m, 8H, ArH), 8.20 (d, J = 9 Hz, 2H, ArH), 13.76 (s, 1H, D₂O exchangeable, NH); MS (C₂₂H₁₇FN₄O₂) m/z = 401 (M⁺).

5-Chloro-3-[3-(4-Methoxyphenyl)-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene] indolin-2-one (9b)

Yield (77%); m.p. 170°C; IR (KBr, cm⁻¹): 3330 (NH), 1665 (C=O); ¹H NMR (DMSO-d₆, δ, ppm): 3.82 (s, 3H, -OCH₃), 7.06 (d, J = 9 Hz, 2H, MeOArH), 7.24 (t, J = 9 Hz, 1H, ArH), 7.45 (t, J = 9 Hz, 2H, ArH), 7.64 (d, J = 9 Hz, 2H, ArH), 7.90 (d, J = 12 Hz, 2H, ArH), 8.00 (d, J = 12 Hz, 2H, ArH), 8.13 (d, J = 12 Hz, 2H, ArH), 13.60 (s, 1H, D₂O exchangeable, NH); MS (C₂₂H₁₇ClN₄O₂) m/z = 500 (M⁺).

5-Chloro-3-[3-(4-Methoxyphenyl)-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene] indolin-2-one (9c)

Yield (65%); m.p. >300°C; IR (KBr, cm⁻¹): 3250 (2NH), 1700-1660 (2C=O); ¹H NMR (DMSO-d₆, δ, ppm): 3.77 (s, 3H, -OCH₃), 6.70-7.95 (m, 8H, ArH), 8.20 (d, J = 9 Hz, 2H, ArH), 13.5 (s, 1H, D₂O exchangeable, NH); MS (C₂₂H₁₇FN₄O₂) m/z = 319 (M⁺).

5-Chloro-3-[3-(4-Methoxyphenyl)-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene] indolin-2-one (9d)

Yield (65%); m.p. >300°C; IR (KBr, cm⁻¹): 3250 (2NH), 1700-1660 (2C=O); ¹H NMR (DMSO-d₆, δ, ppm): 3.77 (s, 3H, -OCH₃), 6.70-7.95 (m, 8H, ArH), 10.45 (s, 1H, D₂O exchangeable, NH isatin), 11.56 (s, 1H, D₂O exchangeable, NH); MS (C₂₂H₁₇FN₄O₂) m/z = 319 (M⁺).
ArH), 6.92-7.89 (m, 5H, 2ArH), 10.63 (s, 1H, D2O exchangeable, NH isatin), 11.23 (s, 1H, D2O exchangeable, NH); MS (C18H12ClN3O3) m/z = 353 (M+).

3-[3-(4-Methoxyphenyl)-5-oxo-1-phenyl-1,5-dihydro-4H-pyrazol-4-ylidene] indolin-2-one (9c)
Yield (72%); m.p. >300°C; IR (KBr, cm⁻¹): 3200 (NH), 1700-1660 (2C=O); ¹H NMR (DMSO-d6, δ, ppm): 3.79 (s, 3H, -OCH3), 6.70-7.98 (m, 13H, 3ArH), 11.20 (s, 1H, D2O exchangeable, NH); MS (C24H17N3O3) m/z = 395 (M+).

5-Chloro-3-[3-(4-methoxyphenyl)-5-oxo-1-phenyl-1,5-dihydro-4H-pyrazol-4-ylidene] indolin-2-one (9d)
Yield (69%); m.p. 240°C; IR (KBr, cm⁻¹): 3150 (NH), 1700-1655 (2C=O); ¹H NMR (DMSO-d6, δ, ppm): 3.80 (s, 3H, -OCH3), 6.65-8.20 (m, 12H, 3ArH), 10.85 (s, 1H, D2O exchangeable, NH); MS (C24H16ClN3O3) m/z = 429 (M+).

Synthesis of 4-[mercapto(phenylamino)methylene]-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one derivatives (10a-b)
To a mixture of compound (4) or (5) (1 mM) and KOH (2 mM) in DMF (25 mL) stirring for 30 min, then 1.1 mM of phenylisothiocyanate were added. The stirring was continued overnight at room temp. Reaction mixture was neutralized with 1 M HCl in ice bath (pH = 7). The desired product was isolated as precipitate after pouring reaction mixture to an ice-cold water. The precipitate was filtered, washed with cold water, dried and recrystallized using absolute ethanol.

4-[Mercapto(phenylamino)methylene]-5-(4-methoxyphenyl)-2,4-dihydro-3H-pyrazol-3-one (10a)
Yield (83%); m.p. 125°C; IR (KBr, cm⁻¹): 3200 (2NH), 1680 (C=O); ¹H NMR (DMSO-d6, δ, ppm): 3.82 (s, 3H, -OCH3), 7.06 (d, J = 9 Hz, 2H, MeOArH), 7.24 (t, J = 9 Hz, 1H, ArH), 7.45 (t, J = 9 Hz, 2H, ArH), 7.64 (d, J = 9 Hz, 2H, ArH), 7.90 (d, J = 12 Hz, 2H, ArH), 8.00 (d, J = 12 Hz, 2H, ArH), 8.13 (d, J = 12 Hz, 2H, ArH), 13.60 (s, 1H, D2O exchangeable, NH); MS (C17H15N3O2S) m/z = 325 (M+).

4-[Mercapto(phenylamino)methylene]-5-(4-methoxyphenyl)-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (10b)
Yield (77%); m.p. 160°C; IR (KBr, cm⁻¹): 3200 (2NH), 1670 (C=O); ¹H NMR (DMSO-d6, δ, ppm): 3.82 (s, 3H, -OCH3), 7.06 (d, J = 9 Hz, 2H, MeOArH), 7.24 (t, J = 9 Hz, 1H, ArH), 7.45 (t, J = 9 Hz, 2H, ArH), 7.64 (d, J = 9 Hz, 2H, ArH), 7.90 (d, J = 12 Hz, 2H, ArH), 8.00 (d, J = 12 Hz, 2H, ArH), 8.13 (d, J = 12 Hz, 2H, ArH), 13.60 (s, 1H, D2O exchangeable, NH); MS (C17H15N3O2S) m/z = 325 (M+).

Scheme 1. Synthesis of pyrazoles 4 and 5
Ar\(\text{H}\), 7.64 (d, \(J = 9\) Hz, 2H, Ar\(\text{H}\)), 7.90 (d, \(J = 12\) Hz, 2H, Ar\(\text{H}\)), 8.00 (d, \(J = 12\) Hz, 2H, Ar\(\text{H}\)), 13.60 (s, 1H, D\(\text{2O}\) exchangeable, NH); MS (C\(_2\)H\(_{19}\)N\(_3\)O\(_2\)S) \(m/z = 401\) (M\(^+\)).

**Biological evaluation**

**In vitro anticancer activity**

Cell culture of HepG-2 (human liver carcinoma), PC-3 (human prostate adenocarcinoma) and HCT116 (human colorectal carcinoma) cell lines were purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium which was supplemented with 10% heat-inactivated FBS (fetal bovine serum), 100 U/mL penicillin and 100 U/mL streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO\(_2\).

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![Graph](image_url)

Figure 1. Anticancer activity of 29 compounds against three cancer types, using MTT assay at 100 µg/mL.

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**Scheme 2. Synthesis of arylidines 6a-j and arylhydrazones 7a-k**
The antitumor activity against HepG-2, PC-3 and HCT-116 human cancer cell lines was estimated using the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells (25-27). Cells were dispensed in a 96 well sterile microplate (5 × 10⁴ cells/well), and incubated at 37°C with series of different concentrations, in DMSO, of each tested compound or doxorubicin (positive control) for 48 h in a serum free medium prior to the MTT assay. After incubation, media were carefully removed, 40 µL of MTT (2.5 mg/mL) were added to each well and then incubated for an additional 4 h. The purple formazan dye crystals were solubilized by the addition of 200 µL of DMSO. The absorbance was measured at 590 nm using a SpectraMax® Paradigm® Multi-Mode microplate reader. The relative cell viability was expressed as the mean percentage of viable cells compared to the untreated control cells.

### Statistical analysis
All experiments were conducted in triplicate and repeated in three different days. All the values

### MTT cytotoxicity assay
The antitumor activity against HepG-2, PC-3 and HCT-116 human cancer cell lines was estimated using the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells (25-27). Cells were dispensed in a 96 well sterile microplate (5 × 10⁴ cells/well), and incubated at 37°C with series of different concentrations, in DMSO, of each tested compound or doxorubicin (positive control) for 48 h in a serum free medium prior to the MTT assay. After incubation, media were carefully removed, 40 µL of MTT (2.5 mg/mL) were added to each well and then incubated for an additional 4 h. The purple formazan dye crystals were solubilized by the addition of 200 µL of DMSO. The absorbance was measured at 590 nm using a SpectraMax® Paradigm® Multi-Mode microplate reader. The relative cell viability was expressed as the mean percentage of viable cells compared to the untreated control cells.

### Statistical analysis
All experiments were conducted in triplicate and repeated in three different days. All the values

### Table 1. The anticancer IC₅₀ values of 29 compounds using MTT assay against the three cancer types.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HCT-116</th>
<th>PC-3</th>
<th>HepG-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (µg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70.06 ± 3.4</td>
<td>86.96 ± 5.7</td>
<td>165.62 ± 5.9</td>
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<tr>
<td>5</td>
<td>66.19 ± 2.9</td>
<td>70.94 ± 3.9</td>
<td>233.42 ± 8.8</td>
</tr>
<tr>
<td>6a</td>
<td>101.29 ± 5.1</td>
<td>172.97 ± 8.1</td>
<td>138.89 ± 6.8</td>
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<tr>
<td>6b</td>
<td>76.41 ± 4.1</td>
<td>871.68 ± 4.8</td>
<td>157.68 ± 7.1</td>
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<tr>
<td>6c</td>
<td>80.19 ± 4.2</td>
<td>128.86 ± 6.9</td>
<td>152.89 ± 6.3</td>
</tr>
<tr>
<td>6d</td>
<td>69.34 ± 3.8</td>
<td>111.25 ± 7.8</td>
<td>245.36 ± 8.9</td>
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<tr>
<td>6e</td>
<td>67.36 ± 2.8</td>
<td>105.31 ± 5.8</td>
<td>165.40 ± 7.3</td>
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<tr>
<td>6f</td>
<td>64.42 ± 5.1</td>
<td>96.54 ± 5.1</td>
<td>218.42 ± 6.9</td>
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<tr>
<td>6g</td>
<td>62.37 ± 4.3</td>
<td>80.55 ± 6.3</td>
<td>258.03 ± 8.1</td>
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<tr>
<td>6h</td>
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<td>74.06 ± 3.8</td>
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<td>59.79 ± 3.9</td>
<td>67.76 ± 4.2</td>
<td>276.11 ± 5.9</td>
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<td>344.39 ± 8.7</td>
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<td>106.67 ± 5.4</td>
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<td>96.00 ± 7.1</td>
<td>&gt; 1000</td>
<td>133.37 ± 5.2</td>
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<td>7d</td>
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<td>167.21 ± 3.2</td>
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<td>7e</td>
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<td>485.15 ± 9.2</td>
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<td>9b</td>
<td>104.42 ± 7.1</td>
<td>&gt; 1000</td>
<td>592.99 ± 9.7</td>
</tr>
<tr>
<td>9c</td>
<td>81.95 ± 4.6</td>
<td>132.79 ± 3.8</td>
<td>322.90 ± 8.9</td>
</tr>
<tr>
<td>9d</td>
<td>78.76 ± 4.3</td>
<td>143.88 ± 6.1</td>
<td>271.83 ± 7.9</td>
</tr>
<tr>
<td>10a</td>
<td>59.12 ± 4.0</td>
<td>68.20 ± 3.1</td>
<td>415.07 ± 9.5</td>
</tr>
<tr>
<td>10b</td>
<td>176.99 ± 8.9</td>
<td>&gt; 1000</td>
<td>216.92 ± 7.1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>73.50 ± 2.9</td>
<td>75.24 ± 4.1</td>
<td>67.9 ± 3.2</td>
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</table>
were represented as the mean ± SD. IC_{50}s were determined by probit analysis using SPSS software program (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Chemistry

The synthetic approach was confined to three general schemes to obtain the target compounds. The reaction of 4-methoxyacetophenone (1) and diethyl carbonate (2) in the presence of sodium hydride afforded the ethyl 3-(4-methoxyphenyl)-3-oxopropanoate (3). Compound 3 undergo smooth reaction with hydrazine hydrate or phenylhydrazine in heated ethanol or glacial acetic acid for 10 min to yield pyrazoles 4 and 5, respectively (Scheme 1). From the literature data, keto form of similar compounds exists in isolated solid compound or in low polarity solvents while hydroxyl form can be preferred in polar solvent such as DMSO (24, 28). \(^1\)H NMR of pyrazoles 4 (R = H) and 5 (R = Ph) in DMSO, showed singlet signal corresponding to C = H at \(\delta 5.8-5.9\) ppm which support the presence of compounds 4 and 5 in enol form (Scheme 1) where IR of these compounds were carried out in solid form KBr adapting disc technique.

Furthermore, the active methylene group of pyrazole ring of compounds 4 and 5 is a favorable unit to react with electrophiles usually resulting in the formation of C= C in case of aldehydes or C=N bond in case of dizonium chloride of anilines. Therefore, the reactivity of compounds 4 and 5 towards substituted aldehydes as carbon electrophiles and toward substituted dizonium chloride of anilines were utilized. The treatment of compound 4 or 5 with a series of aldehydes in refluxed absolute ethanol furnished the corresponding 4-(2-arylidenylidene)-5-(4-methoxyphenyl)-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (6a-j) (Scheme 2).

The reaction of compound 4 or 5 with 2-benzylidenemalononitrile in refluxed absolute ethanol in the presence of piperdine, two expected routes suggested for the reaction product via cyclization or arylidine-exchange via losing of malononitrile, respectively, was leading to either 6-amino-3-(4-methoxyphenyl)-4-phenyl-1,4-dihydropyran[2,3-c]pyrazole-5-carbonitrile structure 8a-j (29, 30) or 6a-j (31, 32), as outlined in Scheme 2. However in all cases, the reaction preceeded via loss of malonitrile according to the proposed mechanism which is shown in Scheme 2, and the reaction product was proved by (IR, MS, \(^1\)H NMR).

Furthermore, the treatment of compound 4 or 5 with dizonium chloride salts of anilines in the presence of anhydrous sodium acetate in ice bath yielded the corresponds 4-(2-(aryl)hydrazineylidene)-5-(4-methoxyphenyl)-2-phenyl-2,4-dihydro-3H-pyrazol-3-ones (7a-j), respectively. The structures of the synthesized compounds were confirmed on the basis of their IR, mass and \(^1\)H NMR spectroscopic analysis (Scheme 2). \(^1\)H NMR of compounds 7a-j showed the appearance of D_{2}O-exchangeable hydrazone NH as a singlet signal which demonstrates the presence of these compounds in the form of hydrazone structure (23, 25) and excludes the possible azo/hydrazo tautomerization.

Moreover, compound 4 or 5 allowed to react with isatin derivatives in refluxing acetic acid in the presence of anhydrous sodium acetate, furnished a single product that was identified as 5-substituted-3-(3-(3-(4-methoxyphenyl)-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene)indolin-2-one 9a-d.

In addition, the reaction of compound 4 or 5 with phenylisothiocyanate in DMF, in the presence of KOH, at room temperature afforded 4-mercap-
to(phenylamino)methylene-5-(4-methoxyphenyl)-2,2,4-dihydro-3H-pyrazol-3-ones \(10a-b\) (Scheme 3).

**Biological activity**

**Anti-tumor activity**

The compounds were examined in vitro for their anti-tumor activities against HepG-2, PC-3 and HCT-116 human carcinoma cell lines using MTT assay. The percentage of the intact cells was measured and compared to the control (Fig. 1). The activities of these compounds against the three carcinoma cells were compared with that of doxorubicin. The obtained results showed that all compounds showed dose-dependent anticancer activities against the three cancer cells.

From Figure 1 we can deduce that, at 100 \(\mu\)g/mL, eight compounds \(6f, 7i, 7h\) showed good anticancer activities against HCT-116 carcinoma cells. Fifteen compounds \(6f, 7g, 5, 7h, 6e, 7f, 6d, 4, 6h, 6b, 7d, 9d, 7c, 12b, 7e\) showed moderate activities and the rest of the compounds showed weak activities against HCT-116 cells. In addition, five compounds \(7a, 6i, 10a, 5, 7k\) showed good anticancer activities, six compounds \(6h, 7j, 6g, 7i, 6j, 4\) showed moderate antitumor activity and the rest of the compounds showed weak or no antitumor activities against PC-3 cancer cells. Furthermore, all the compounds showed weak or no anticancer activities against HepG-2 liver cancer. The IC\(_{50}\) values are shown in Table 1.

Structure–activity relationships in structures \(6a-j, 7a-k, 9a-d\) and \(10-b\) demonstrated that compounds with \(para\) electron-withdrawing substituent \((-N(CH_3)_2, F, Cl, Br\) showed higher anticancer activities than those with electron-donating substituent \((-CH_3, -OCH_3)\). A comparison of the \(para\) substituent demonstrated that an electron-withdrawing group has improved anticancer activity and the potency order is \(-N(CH_3)_2 > F > Cl > Br\), whereas the donating group substituent had moderate effects in the case of \(-N-\text{Ph}\) and had weak or no effects in the case of \(-N-H\). The derivatives with \(-N-\text{Ph}\) showed stronger anticancer activities than \(-N-H\) of the compounds.

**CONCLUSION**

In summary, we synthesized a series of pyrazole derivatives and their structures confirmed by IR, mass and NMR spectroscopic analysis. These compounds were examined for their anticancer activities on three different human tumor cell lines. The results showed that the compounds \(7a, 10b, 6i, 7k, 6j, 7j, 6g, 7i\) showed good anticancer activities against HCT-116 carcinoma cells. The compounds \(7a, 6i, 10a, 5, 7k\) showed good anticancer activities against PC-3 cancer cells and all the compounds showed weak or no anticancer activities against HepG-2 liver cancer.

**REFERENCES**


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SYNTHESIS AND DESIGN OF NEW BROMOQUINAZOLINE DERIVATIVES WITH ANTI-BREAST CANCER ACTIVITY

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Abstract: A new series of 6- bromoquinazolin-4(3H)-one derivatives were synthesized. Their chemical structures were confirmed by spectral and elemental analysis. All synthesized compounds were tested in vitro against human breast cancer cell line (MCF-7). Compounds IIIc, Vb and IIla exerted powerful cytotoxic activity with IC50 (0.236, 0.316 and 0.359 µM/mL), respectively.

Keywords: quinazolinone; anti-tumor, breast cancer

Quinazoline is considered one of the most important scaffolds in medicinal chemistry due to its diverse biological activities. Several quinazoline derivatives possess anticancer (1-9), antihypertensive (10), antiviral (11, 12), sedative (13), antibacterial (14-20), anti-inflammatory and analgesic activities (21-30). Hybridization of quinazolin-4-one with 2-imino pyridine, compound 1 (Fig. 1) shows potent anticancer activity (31). While hybridization with pyran-3-carbonitrile gives compound 2 with cytotoxic activity higher than doxorubicin (31). 2,3 disubstituted quinazoline moiety (compound 3) have promising anticancer activity (32) against MCF-7 cell line (Fig. 1).

Dibromoquinazoline derivatives bearing 4-chlorophenyl at position 2 and aniline derivatives at position 3 (compound 4) exert powerful anti-breast cancer activity (33). In continuation of our research program (28-36), our target in this work is to hybridize pyran or pyridine moiety with bromo quinazoline derivative bearing 4-chlorophenyl moiety at position 2 and aniline moiety at position 3 hopping to obtain safe and potent anticancer derivatives against MCF-7 cell line.

EXPERIMENTAL

Chemistry

All melting points are measured using Electro-thermal IA 9100 apparatus (Shimadzu, Japan). IR spectra were recorded on Perkin Elmer 1650 spectrophotometer (USA), in Faculty of Science, Cairo University, Cairo, Egypt. 1H NMR spectra were determined on JOEL NMR FXQ-300 MHz using TMS as an internal standard. 13C NMR (DMSO-d6) spectra were recorded at 100.62 MHz at the aforementioned research center in Cairo University. Mass spectra were determined on at 70 eV EI Ms-QP 1000 EX (Shimadzu, Japan). Microanalyses were operated using Vario EL elemental apparatus (Shimadzu, Japan).

6-[(6-Bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl)[phenyl]-2-oxo-4-(substituted aryl)-1,2-dihydropyridine-3-carbonitrile (IIIa-d)

General method

A mixture of ketone II (0.9 g; 0.002 M), ethyl cyanoacetate (0.23 mL, 0.002 M), anhyd. ammonium acetate (1.24 g; 0.016 M) and the appropriate aldehydes namely, p-chlorobenzaldehyde, 3,4-dichlorobenzaldehyde, 3,4,5- trimethoxybenzaldehyde and/or furan-2-carboxaldehyde in 10 mL n-butanol was refluxed for 6 h. The reaction mixture was concentrated to half its volume under reduced pressure. After cooling, the formed precipitate was filtered off, air dried, and recrystallized from the proper solvent to give compounds IIIa-d, respectively.

6-[(6-Bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl)[phenyl]-2-oxo-1,2-dihydropyridine-3-carbonitrile (IIa)

* Corresponding author: e-mail: marwafarag80@yahoo.com
Crystallized from ethanol to give yellow crystals, m.p. 110°C in 70% yield. Analysis for C₃₂H₁₇BrCl₂N₄O₂, calcd.: C, 60.02; H, 2.68; N, 8.75%. Found: C, 60.17; H, 2.74; N, 8.69%. IR spectrum (KBr, cm⁻¹) showed absorption bands at 3380 (NH), 2225 (-CN), 1745 (C=O, pyridone), 1685 (C=O, quinazolinone), and 1610 (C=C).

'HNMR spectrum (DMSO-d₆, δ, ppm) showed 6.2 (1H, s, 1 H of pyridine), 7.3-8.2 (m, 15H, Ar-H), and at 9.5 (1 H, s, NH proton, exchangeable with D₂O).

¹³C NMR (DMSO-d₆, δ, ppm): 165, 161, 160.5, 157, 149, 136.4, 136, 133.9, 133.6, 132, 131.7, 130, 129.1, 128.9, 128.7, 126.7, 126.6, 126.4, 124.5, 122, 121.7, 116. MS: m/z = 639.

6-{4-[6-Bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(3,4-dichlorophenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (IIIb)

Crystallized from methanol to give yellowish brown crystals, m.p. 115°C in 80% yield. Analysis for C₃₂H₁₆BrCl₃N₄O₂, calcd.: C, 56.96; H, 2.39; N, 8.3%; found: C, 56.89; H, 2.42; N, 8.42%. IR spectrum (KBr, cm⁻¹) showed absorption bands at 3385 (NH), 2235 (-CN), 1750 (C=O, pyridone), 1680 (C=O, quinazolinone), and 1600 (C=C).

'HNMR spectrum (DMSO-d₆, δ, ppm) showed 6.4 (1H, s, 1 H of pyridine), 7.4-8.6 (m, 14H, Ar-H), and at 11.5 (1 H, s, NH, exchangeable with D₂O).


6-{4-[6-Bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(3,4,5-trimethoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (IIIc)

Crystallized from methanol to give yellow crystals, m.p. 195°C in 80% yield. Analysis for C₃₅H₂₄BrClN₄O₅, calcd.: C, 60.40; H, 3.48 N, 8.05%; found: C, 60.50; H, 3.61; N, 8.12%. IR spectrum (KBr, cm⁻¹) showed absorption bands at 3380 (NH), 2225 (-CN), 1765 (C=O, pyridone), 1680 (C=O, quinazolinone), and 1600 (C=C).

'HNMR spectrum (DMSO-d₆, δ, ppm) showed 3.9 (9H, s, OCH₃), 6.4 (1H, s, 1 H of pyridine), 7.2-8.7 (m, 13H, Ar-H) and at 11.5 (1 H, s, NH, exchangeable with D₂O).


6-{4-[6-Bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(furan-2-yl)-2-oxo-1,2-dihydropyridine-3-carbonitrile (IIId)

Crystallized from methanol to give brown crystals, m.p. 195°C in 80% yield. Analysis for C₃₅H₂₄BrClN₄O₅, calcd.: C, 60.40; H, 3.48 N, 8.05%; found: C, 60.50; H, 3.61; N, 8.12%. IR spectrum (KBr, cm⁻¹) showed absorption bands at 3380 (NH), 2225 (-CN), 1765 (C=O, pyridone), 1680 (C=O, quinazolinone), and 1600 (C=C).

'HNMR spectrum (DMSO-d₆, δ, ppm) showed 3.9 (9H, s, OCH₃), 6.4 (1H, s, 1 H of pyridine), 7.2-8.7 (m, 13H, Ar-H) and at 11.5 (1 H, s, NH, exchangeable with D₂O).

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C\textsubscript{30}H\textsubscript{16}BrClN\textsubscript{4}O\textsubscript{3}, calcd.: C, 60.47; H, 2.78; N, 9.47%; found: C, 60.57; H, 2.83; N, 9.52%. IR spectrum (KBr, cm\textsuperscript{-1}) showed absorption bands at 3370 (NH), 2220 (5-CN), 1755 (C=O, pyridone), 1670 (C=O, quinazolinone) and 1595 (C=C). 1HNMR spectrum (DMSO-d\textsubscript{6}, \(\delta\), ppm) showed 6.4 (1H, s, 1 H of pyridine), 7.1-8.4 (m, 14H, Ar-H) and at 10.0 (1 H, s, NH, exchangeable with D\textsubscript{2}O). 13C NMR (DMSO-d\textsubscript{6}, \(\delta\), ppm): 169.5, 164, 160.6, 160.1, 159.5, 149.9, 147.7, 143.7, 136.3, 135.7, 132.2, 131, 129, 128.8, 126.7, 126.6, 124, 124.2, 122, 121.7, 121.3, 117, 113, 110. MS: m/z = 596.

6-{4-[(6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl)]-2-imino-1,2-dihydropyridine-3-carbonitrile (IVa-d)

General method

A mixture of compound II (0.9 g, 0.002 M), malononitrile (0.12 mL, 0.002 M), anhyd. ammonium acetate (1.24 g, 0.016 M) and the appropriate aldehydes namely, p-chlorobenzaldehyde, 3,4-dichlorobenzaldehyde, 3,4,5-trimethoxybenzaldehyde and/or furan-2-carboxaldehyde in 10 mL n-butanol was refluxed for 6 h. After cooling, the reaction mixture was filtered and the precipitate was crystallized from the proper solvent to give the iminopyridines IV a-d, respectively.

6-{4-[(6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl)]-4-(4-chlorophenyl)-2-imino-1,2-dihydropyridine-3-carbonitrile (IVa)

Crystallized from glacial acetic acid to give red crystals, m.p. 145°C in 75% yield. Analysis for C\textsubscript{32}H\textsubscript{18}BrCl\textsubscript{2}N\textsubscript{5}O, calcd.: C, 60.12; H, 2.84; N, 10.95%; found: C, 60.25; H, 2.92; N, 10.99%. IR spectrum (KBr, cm\textsuperscript{-1}) absorption bands at 3335-3250 (NH, =NH), 2215 (C=N), 1710 (C=O, quinazolinone) and at 1600 (C=N). 1HNMR spectrum (DMSO-d\textsubscript{6}, \(\delta\), ppm) showed 7.4-8.3 (m, 16H, Ar-H) and 9.70, 11.60 (2H, 2s, 2NH, exchangeable with D\textsubscript{2}O). 13C NMR (DMSO-d\textsubscript{6}, \(\delta\), ppm): 170, 167, 163, 162, 160, 148, 136.4, 135.7, 133.9, 133, 132, 130, 129.1, 128.9, 128.7, 126.7, 126.5, 126.3, 124.6, 124.3, 123, 121, 118, 115, 103. MS: m/z = 639.

6-{4-[(6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl)]-4-(3,4-dichlorophenyl)-2-imino-1,2-dihydropyridine-3-carbonitrile (IVb)

Crystallized from methanol to give reddish brown crystals, m.p. 132°C in 75% yield. Analysis for C\textsubscript{32}H\textsubscript{17}BrCl\textsubscript{3}N\textsubscript{5}O, calcd.: C, 57.04; H, 2.54%; N, 10.39; found: C, 57.15; H, 2.62; N, 10.41%. IR spectrum (KBr, cm\textsuperscript{-1}) absorption bands at 3335-3215 (NH, =NH), 2210 (C=N), 1720 (C=O, quinazolinone), and at 1610 (C=N). 1HNMR spectrum (DMSO-d\textsubscript{6}, \(\delta\), ppm) showed 7.6-8.5 (m, 15H, Ar-H) and 10.60, 12.30 (2H, 2s, 2NH, exchangeable with D\textsubscript{2}O). 13C NMR (DMSO-d\textsubscript{6}, \(\delta\), ppm): 173, 164, 162, 161, 148, 136.2, 133, 133.3, 132.6, 132.3, 132.1, 130.1, 129.2, 128.7, 128, 127.6, 126.5, 124.6, 124.4, 124.3, 123, 121, 118.5, 116, 105. MS: m/z = 672.97.

6-{4-[(6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl)]-4-(3,4,5-trimethoxyphenyl)-2-imino-1,2-dihydropyridine-3-carbonitrile (IVc)

Crystallized from glacial acetic acid to give brown crystals, m.p. 215°C in 70% yield. Analysis for C\textsubscript{32}H\textsubscript{17}BrCl\textsubscript{3}N\textsubscript{5}O, calcd.: C, 60.49; H, 3.63%; N, 10.08; found: C, 60.52; H, 3.67; N, 10.10%. IR spectrum (KBr, cm\textsuperscript{-1}) absorption bands at 3335-3220 (NH, =NH), 2200 (C=N), 1720 (C=O, quinazolinone) and at 1610 (C=N). 1HNMR spectrum (DMSO-d\textsubscript{6}, \(\delta\), ppm) showed 3.8 (9H, s, OCH\textsubscript{3}), 6.55-8.4 (m, 14H,) and 9.70, 11.90 (2H, 2s, 2NH, exchangeable with D\textsubscript{2}O). 13C NMR (DMSO-d\textsubscript{6}, \(\delta\), ppm): 176, 166, 165, 163, 150, 139, 137, 136, 135, 132, 129, 126.8, 126.7, 126.6, 126.4, 124.5, 124.2, 123, 119, 117, 60, 56. MS: m/z = 695.

6-{4-[(6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl)]-4-(furan-2-yl)-2-imino-1,2-dihydropyridine-3-carbonitrile (IVd)

Crystallized from ethanol to give brown crystals, m.p. 240°C in 75% yield. Analysis for C\textsubscript{30}H\textsubscript{17}BrClN\textsubscript{5}O\textsubscript{2}, calcd.: C, 69.84; H, 3.52%; N, 11.77; found: C, 69.79; H, 3.57; N, 11.64%. IR spectrum (KBr, cm\textsuperscript{-1}) absorption bands at 3320-3210 (NH, =NH), 2200 (C=N), 1710 (C=N), 1600 (C=N). 1HNMR spectrum (DMSO-d\textsubscript{6}, \(\delta\), ppm) showed 6.9-8.4 (m, 15H, Ar-H) and 9.70, 11.20 (2H, 2s, 2NH, exchangeable with D\textsubscript{2}O). 13C NMR (DMSO-d\textsubscript{6}, \(\delta\), ppm): 169.5, 164, 162, 150.1, 147.7, 144, 137, 135, 132.3, 131.7, 129, 128.9, 126.9, 126.7, 126.4, 124.3, 122, 121, 115, 113, 109. MS: m/z = 595.
Table 1. Eight dose growth inhibition percent and IC₅₀ values of the test compounds against MCF-7 cell line.

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2-Amino-6-{4-[6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(4-chlorophenyl)-4H-pyran-3-carbonitrile (Va)

Crystallized from methanol to give yellow crystals, m.p. 176°C in 75% yield. Analysis for C₃₂H₁₉BrCl₂N₄O₂, calcd.: C, 59.84; H, 2.98%; N, 8.72; found: C, 59.72; H, 2.95; N, 8.80%. IR spectrum (KBr, cm⁻¹) absorption bands at 3435-3360 (NH₂), 2225 (-CN), 1685 (C=O quinazolinone), and at 1600 (C=O). ¹H NMR spectrum (DMSO-d₆, δ ppm) 4.1 (1H, d, 1 H of pyran), 5.1 (1H, d, 1 H of pyran), 7.5-8.4 (m, 15H, Ar-H) and 9.8 (2H, s, NH₂, exchangeable with D₂O). ¹³C NMR (DMSO-d₆, δ ppm): 168, 162, 160, 149, 140, 140.2, 136.7, 136.6, 135.8, 132.2, 132.1, 130.4, 129.1, 128.9, 128.6, 128.4, 126.7, 126, 125.9, 124.8, 124.5, 123, 121.7, 120, 91.5, 59, 30 MS: m/z = 642.

2-Amino-6-{4-[6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(3,4-dichlorophenyl)-4H-pyran-3-carbonitrile (Vb)

Crystallized from ethanol to give brown crystals, m.p. 215°C in 70% yield. Analysis for C₃₂H₁₈BrCl₃N₄O₂, calcd.: C, 56.79; H, 2.68; N, 8.28%; found: %C, 56.79; H, 2.68; N, 8.39%. IR spectrum (KBr, cm⁻¹) absorption bands at 3440-3360 (NH₂), 2220 (-CN), 1690 (C=O quinazolinone), and at 1610 (C=O). ¹H NMR spectrum (DMSO-d₆, δ ppm) showed 4.5 (1H, d, 1 H of pyran), 5.4 (1H, d, 1 H of pyran), 7.5-8.4 (m, 14H, Ar-H) and 10.1 (2H, s, NH₂, exchangeable with D₂O). ¹³C NMR (DMSO-d₆, δ ppm): 169, 163, 157, 148, 143, 141, 139, 136, 132.8, 131.9, 130.5, 130.2, 130, 129, 128.9, 128.5, 126.7, 126, 124.4, 124, 122, 119, 92, 60, 32. MS: m/z = 677.

2-Amino-6-{4-[6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(3,4,5-trimethoxyphenyl)-4H-pyran-3-carbonitrile (Vc)

Crystallized from glacial acetic acid to give yellow crystals, m.p. 240°C in 70%
Figure 2. Cell viability curves of all synthesized compounds
yield. Analysis for C_{35}H_{26}BrClN_{4}O_{5}, calcd.: C, 60.23; H, 3.75; N, 8.03%; found: C, 60.35; H, 3.80; N, 8.12%. IR spectrum (KBr, cm\(^{-1}\)) absorption bands at 3430-3350 (NH\(_2\)), 2220 (-CN), 1685 (C=O quinazolinone), and at 1610 (C=N). 

1HNMR spectrum (DMSO-d\(_6\), \(\delta\), ppm) showed 3.8 (9H, s, OCH\(_3\)), 4.2 (1H, d, 1 H of pyran), 5.1 (1H, d, 1 H of pyran), 7.2-8.3 (m, 13H, Ar-H) and 9.0 (2H, s, NH\(_2\), exchangeable with D\(_2\)O). 13C NMR (DMSO-d\(_6\), \(\delta\), ppm): 165, 161, 159.9, 152.9, 148, 141, 136.5, 136.3, 136, 135.5, 132.3, 131.9, 130, 129.1, 127, 126.6, 125.9, 124.6, 124.3, 123, 122, 119, 106, 91, 61, 56.2, 29.3. MS: m/z = 698.

2-Amino-6-{4-[6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(furan-2-yl)-4H-pyran-3-carbonitrile (Vd)

Crystallized from ethanol to give yellow crystals, m.p. 180°C in 70% yield. Analysis for C\(_{30}\)H\(_{18}\)BrClN\(_{4}\)O\(_{3}\), calcd.: C, 60.27; H, 3.03; N, 9.37%; found: C, 60.31; H, 3.12; N, 9.30%. IR spectrum (KBr, cm\(^{-1}\)) absorption bands at 3420-3350 (NH\(_2\)), 2200 (-CN), 1670 (C=O quinazolinone), and at 1600 (C=N). 1HNMR spectrum (DMSO-d\(_6\), \(\delta\), ppm) showed 4.0 (1H, d, 1 H of pyran), 4.9 (1H, d, 1 H of pyran), 7.0-8.2 (m, 14H, Ar-H) and 9.4 (2H, s, NH\(_2\), exchangeable with D\(_2\)O). 13C NMR (DMSO-d\(_6\), \(\delta\), ppm): 163, 160.5, 158, 152.5, 148, 143, 140.5, 136.4, 132.3, 131.9, 129, 128.5, 126.6, 126.4, 125.9, 124.6, 124.3, 123, 119, 90, 31. MS: m/z = found: 597.

Pharmacological screening

Cell line propagation

The cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 1% L-glutamine, 50 µg/mL gentamycin (37), HEPES buffer and 10% heat-inactivated fetal

Scheme 1. Synthesis of quinazoline derivatives IIIa-d
bovine serum. All cells were maintained in a humidified atmosphere at 37°C with 5% CO₂ and were subcultured two times a week. Cell toxicity was monitored by determining the effect of the test samples on cell viability and cell morphology.

**Cytotoxicity evaluation using viability assay**

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1 × 10⁴ cells per well in 100 µL of growth medium (37, 38). After 24 h of seeding fresh medium containing different concentrations of the test sample was added. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO₂ for a period of 48 h. Control cells were incubated with or without DMSO and without test sample. After incubation of the cells for at 37°C, various concentrations of sample were added, and the incubation was continued for 24 h and viable cells yield was determined by a colorimetric method.

After the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added for at least 30 min. The plates were rinsed using tap water. Glacial acetic acid (30%) was then added and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated.

**RESULTS AND DISCUSSION**

**Chemistry**

Compounds I and II are synthesized according to reported method (18). One pot reaction of ketone II with aromatic aldehydes, namely, p-chlorobenzaldehyde, 3,4-dichlorobenzaldehyde, 3,4,5-trimethoxybenzaldehyde and/or furan-2-carboxaldehyde with ethyl cyanoacetate afforded the corresponding pyridine-2(1H)-ones III a-d, respectively (Scheme 1). The one pot reaction of II with the same aldehydes and malononitrile in the presence of anhyd. ammonium acetate give the corresponding 2(1H)-iminopyridine derivatives IVa-d, respectively.

Scheme 2. Synthesis of quinazolin-4-one derivatives IVa-d, Va-d
(Scheme 2), while the same one pot reaction of II with malononitrile in piperidine, gave the corresponding 2-aminopyrans Va-d (Scheme 2).

**Biological activity**

*In vitro cytotoxic activity*

In this study a novel series of 6-bromo-2-(4-chlorophenyl)quinazolin-4(3H)-one derivatives were synthesized (Schemes 1, 2). All synthesized compounds were screened for their *in vitro* cytotoxic activities against MCF-7 cell line. Growth curves showed that most our tested compounds exhibit significant efficacy against MCF-7 with IC₅₀ values range (0.236 – 0.637 µMol/mL). As shown in Table 1: compounds IIIc, Vb and IIIa exerted the most potent cytotoxic effect against MCF-7 with IC₅₀ (0.236, 0.316 and 0.359 µM/mL) respectively. Compounds IVa, IVd, IIIb, Vd and IIId exerted a moderate cytotoxic effect (IC₅₀ 0.417, 0.420, 0.444, 0.474, 0.557 and 0.637 µMol/mL, respectively), while compounds IVb, IVc, and Vc exerted a weak cytotoxic effect against MCF-7.

**Structure - Activity Relationships**

Concerning 6-{4-[6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-2-oxo-4-(substituted aryl)-1,2-dihydropyridine-3-carbonitrile (IIId-d) derivatives, substitution with 4-chlorophenyl IIIa, or 3,4,5- trimethoxyphenyl IIIc at position 4 gives the most potent cytotoxic activity. While substitution at the same position with 3,4-dichlorophenyl IIIb, or with furyl moiety IIId gives moderate cytotoxic effect.

On discussing the SAR of compounds 6-{4-[6-bromo-2-(4-chlorophenyl)-4-oxoquinazolin-3(4H)-yl]phenyl}-2-imino-4-(substituted aryl)phenyl-1,2-dihydropyridine-3-carbonitrile (IVA-d), substitution with 4-chlorophenyl IVa, or furyl moiety IVd gives moderate cytotoxic effect. While substitution with 3,4-dichlorophenyl IVb, or with furyl moiety IVd decreases the cytotoxic activity.

On the other hand, substitution of 2-amino-6-[4-{4-[2-(furan-2-yl)-4-oxoquinazolin-3(4H)-yl]phenyl}-4-(4-substituted phenyl)-4H-pyran-3-carbonitrile with 3,4-dichlorophenyl Va, or with furyl moiety Vd gives potent cytotoxic effect, while substitution with 4-chlorophenyl Va, or furyl moiety Vd gives moderate cytotoxic effect. Substitution with 3,4,5- trimethoxyphenyl VC decreases the cytotoxic activity.

**CONCLUSION**

In summary, 6 bromo-2-(4-chlorophenyl)quinazolin-4(3H)-one derivatives III-V were synthesized. All synthesized compounds were tested *in vitro* against human breast cancer cell line (MCF-7). Compounds IIIc, Vb and IIIa exerted powerful cytotoxic activity with IC₅₀ (0.236, 0.316 and 0.359 µM/mL) respectively.

**Acknowledgement**

I deeply thank Ghadeer Manei Aljuaid (Lab technician) and Sameerah Eid Alharthi (Lab technician) faculty of pharmacy Taif University, for lab facilities and kind help during the research.

**REFERENCES**


Received: 19. 09. 2016
Tuberculosis still remains a major public health threat despite years of research. The World Health Organization (WHO) estimates that TB is the leading infectious cause of death worldwide. Moreover, TB accounts for the loss of two million lives annually as a result of e.g., presence of multidrug-resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis*. In the last decade, there has been growing interest in developing new substances that are efficient against *M. tuberculosis*. Among them, there areazole derivatives widely known as antifungal agents. Nowadays, some bicyclic nitroimidazoles (PA-824, CGI-17341, OPC-67683) undergoing clinical trials as promising tuberculostatic agents. In our study, bicyclic 7-nitroimidazo[5,1-b]2,3-dihydrooxazoles, isomeric with CGI-17341 and 3-hydroxy-8-nitroimidazo[5,1-b]-1,4,5,6-tetrahydropyrimidines, which are structural isomers of the structure of PA-824 were synthesized and tested for their physicochemical properties. It has turned out that there are interesting differences between bicyclic isomers. In order to explain the reasons of the experimental disparities in biological activity of tested compounds and referenced nitroimidazooxazines PA-824, it was decided to carry out a molecular docking simulations.

Keywords: tuberculosi, antitubercular activity, azoles, bicyclic nitroimidazoles, molecular docking simulations

Tuberculosis still remains a major public health threat despite years of research. The World Health Organization (WHO) estimates that TB is the leading infectious cause of death worldwide. Moreover, TB accounts for the loss of two million lives annually as a result of e.g., presence of multidrug-resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis*. In the last decade, there has been growing interest in developing new substances that are efficient against *M. tuberculosis*. Among them, there areazole derivatives widely known as antifungal agents. Nowadays, some bicyclic nitroimidazoles (PA-824, CGI-17341, OPC-67683) undergoing clinical trials as promising tuberculostatic agents. In our study, bicyclic 7-nitroimidazo[5,1-b]2,3-dihydrooxazoles, isomeric with CGI-17341 and 3-hydroxy-8-nitroimidazo[5,1-b]-1,4,5,6-tetrahydropyrimidines, which are structural isomers of the structure of PA-824 were synthesized and tested for their physicochemical properties. It has turned out that there are interesting differences between bicyclic isomers. In order to explain the reasons of the experimental disparities in biological activity of tested compounds and referenced nitroimidazooxazine PA-824, it was decided to carry out a molecular docking simulations.

Keywords: tuberculosi, antitubercular activity, azoles, bicyclic nitroimidazoles, molecular docking simulations
Table 1. Physicochemical parameters for bicyclic nitroimidazole derivatives.

![Diagram of bicyclic nitroimidazole derivatives]

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3: R = -H, R1 = -CH2CH3  
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6: R = -CH3, R1 = -Ph  
7: R = -H, R1 = -CH2Br  
8: R = -CH3, R1 = -CH2Br  
9: R = -H, R1 = -CH2O-i-Pr  
10: R = -CH3, R1 = -CH2O-i-Pr  
11: R = -H, R1 = -Ph  
12: R = -CH3, R1 = -Ph  
13: R = -H, R1 = -C6H4Cl  
14: R = -CH3, R1 = -C6H4Cl  
15: R = -H, R1 = -C6H4Br  
16: R = -CH3, R1 = -C6H4Br  
17: R = -H, R1 = -C6H4I  
18: R = -CH3, R1 = -C6H4I  
19: R = -H, R1 = -(CH2)3CH3  
20: R = -CH3, R1 = -(CH2)3CH3  
21: R = -H, R1 = -CH2CH(CH3)3  
22: R = -CH3, R1 = -CH2CH(CH3)3
These observations encouraged us to synthesize a set of close structural analogs to CGI-17341 and PA-824.

EXPERIMENTAL

Bicyclic 7-nitroimidazo[5,1-b]-2,3-dihydrooxazoles, isomeric with CGI-17341 and 3-hydroxy-8-nitroimidazo[5,1-b]-1,4,5,6-tetrahydropyrimidines, which are structural isomers of the structure of PA-824 were synthesized as it was described in (14) and (15).

Density functional calculations were executed and the geometries of referenced PA-824 and representative compound 14 were optimized at the DFT level of theory using the Gaussian 09 D.01 program (16), B3LYP functional, 6-31G(d,p) basis set, and Conductor-like Polarizable Continuum Model (CPCM, water as a solvent) (17-19). The vibrational frequencies and thermodynamic properties were calculated by applying the ideal gas, rigid rotor, and harmonic oscillator approximations, energy minimum was confirmed by the frequency calculation for all conformers, no negative frequencies were detected in generated vibrational spectrum of analyzed conformers. The binding affinity of the analogue 14 were obtained using AutoDock Vina tool (20). Water molecules were excluded from target active site.

RESULTS

As it is shown in Table 1, the obtained bicyclic nitroimidazoles, in most cases, are fully compatible with Lipinski’s rule of five (21). They have favorable values of C log P (< 5), molecular weight less than 500 Daltons, no more than 10 hydrogen bond acceptors and 5 H-bond donors, low and moderate TPSA parameters.

The results listed in Table 1 show good molecular properties, similar to those that are characteristic for compounds PA-824, CGI-17341 and OPC-67683. Although lipophilicity values calculated for obtained compounds are rather favorable, both 7-nitroimidazo[5,1-b]-2,3-dihydrooxazoles and 3-hydroxy-8-nitroimidazo[5,1-b]-1,4,5,6-tetrahydropyrimidines do not exhibit in vitro activity against M. tuberculosis H37Rv in tested concentrations (14, 15). Results for selected products are collected in Table 2.

There have to be some other additional factors like steric and electronic, which are responsible for appearance of tuberculostatic activity. In our previous papers (14,15) we have taken the attempts of
DISCUSSION AND CONCLUSION

It is known from the literature (22) that there are some key structural features that are important for aerobic activity: the preference for the 4-nitroimidazole, rigid bicyclic system, electron-donating component at the 2-position in imidazole ring and the presence of hydrophobic substituent at six-membered saturated ring condensed with imidazole. In case of 8-nitroimidazo[5,1-b]tetrahydropyrimidines, first and second condition was realized. Probably, the most essential requirement is the nature of the substituent at the C-2 position of the imidazole ring. The presence of alkyl group or hydrogen atom at this position significantly reduces anti-Mtb activity (22). Considering the structural features of nitroimidazo[2,1-b]dihydrooxazoles having tuberculostatic potency, it can be found some key factors: 4-nitroderivatives are more active, the presence of small,
lipophilic alkyl groups or alkyl halides as substituents in saturated oxazole ring and oxygen atom at C-2 position of imidazole ring (23). In case of isomeric nitroimidazo[5,1-b]dihydrooxazoles, electron-donating atom is placed at C-5 position of imidazole ring. It seems that it is the essential hindrance in appearance of tuberculostatic activity among the bicyclic nitroimidazo[5,1-b]dihydrooxazole class.

Additionally, in order to explain the reasons of the experimental differences in biological activity of tested compounds and referenced nitroimidazooxazine PA-824, it was decided to carry out a molecular docking simulations. In computational medicinal chemistry, some of the most helpful information is obtained by visualising the 3D structures of proteins, chemical compounds, and protein – ligand docking poses (6). Public databases containing the 3D conformations of proteins are widely used for \textit{in silico} techniques. On the base of the data known from the literature (24), the potential target binding site has been identified – Deazaflavin-dependent nitroreductase (Ddn) from \textit{M. tuberculosis} involved in bioreductive activation of PA-824 (24). Also, it is known, that the most potent aminoacids within of Ddn structure able to interact with PA-824 are: Tyr-130, Tyr-136, Ser-78, and Trp-20. In this analysis, nine conformers has been generated. It was found that only in four cases overlapping of PA-824 and compound 14 is observed (Fig. 2).

Moreover, the energy value of the affinity (binding mode) of compound 14 is slightly higher comparing with PA-824: -5.2 and -5.6 kcal/M, respectively (Table 3).

Also, the range of energy between the most preferred to the less stable conformer is higher in the case of 14 than in PA-824 (1.3 and 0.4 kcal/M), respectively. It indicates that compound 14 is less stable than PA-824. The better efficacy of PA-824 and Ddn bonding in comparison with analogue 14 is also connected with higher lipophilicity (log P) of

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native ligand (mi log P = 3.977 and 1.983, respectively). Similar trend is observed for log P that has been determined with quantum methods in SMD model (25) (0.726 and 0.243, respectively). It is clear that PA-824 is better penetrating through lipophilic cell membranes, that is why it shows higher biological activity, than tested ligand 14.

REFERENCES


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SYNTHESIS AND ANTIMICROBIAL EVALUATION OF SOME
NEW QUINALDINE DERIVATIVES

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Assiut 71516, Egypt

Abstract: A new series of quinoline derivatives incorporated to glycosides and biologically active heterocyclic
moieties were synthesized starting with 6-bromo-4-hydroxyquinaldine (6-bromo-4-hydroxy-2-methylquinoline)
compound 1. Some of the synthesized compounds were evaluated for their antibacterial and antifungal
activities. Most of the tested compounds exhibited potential antibacterial activity against Gram-positive bacte-
ria, the detailed synthesis, spectroscopic data, antibacterial and antifungal evaluation of the synthesized com-
pounds are reported.

Keywords: quinaldine derivatives, selenadiazole, imidazole, glycosides, antibacterial, antifungal

The number of life threatening infectious dis-
eases and microbial multidrug-resistance are respon-
sible for great number of deaths all over the world.
Drug discovery programs have reported that many
natural and synthetic products are classified as
antimicrobial agents. Quinoline derivatives which
present in several natural products (Cinchona alka-
loids) produce diverse biological and pharmacolog-
ical activities such as; antimalarial (1-3), anticancer
(4-6), antituberculosis (7, 8), anti-inflammatory (9,
10) analgesic (11), HIV-1 integrase inhibitors (12),
antioxidant (13), anti-depressant (14), and anti-con-
vulsant (15). Till date, this class plays an essential
role in the field of antibiotic chemotherapy used for
the treatment of a large number of serious bacterial
infections (16-18). Many hypotheses have been
advanced to account quinoline’s mode of actions
(19-21). These have included DNA binding by inter-
calation quinoline drug with the microbial DNA
thus, inhibits nucleic acid synthesis, replication and
transcription (22).

The most common powerful quinoline deriva-
tives as antibiotics are fluoroquinolones (Fig. 1).
However, literature survey reported that most of
them have adverse effects which may occur almost
anywhere in the body. Most of fluoroquinolone
drugs have fluoride atom as a central part which has
the unique ability to penetrate the blood-brain barri-
er, entering the brain and causing undesirable
effects. One of the most common adverse effects of
this type of antibiotic is disturbances of the CNS
(23, 24). So, the main challenge that always faces
the researchers is discovering and developing new
antimicrobial agents without or with the lowest
adverse effects to treat serious microbial infections.
Depending upon the above knowledge and in con-
tinuation to our previous efforts dealing with syn-
thesis of various 6-bromoquinaldine derivatives (25,
26) of strong antimicrobial potency, the aim of this
study is to synthesize some new 6-bromoquinaldine
derivatives incorporated to different biologically
active heterocycles that possess broad spectrum
antimicrobial potency like, imidazole (27, 28), pyra-
zole (29, 30), furane (31, 32) or to glycosidic moi-
etes of reported antimicrobial activity through S- or
N-linkages (33, 34) to evaluate their antimicrobial

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activity against a number of Gram positive, Gram negative bacteria as well as fungi. The obtained biological results have been summarized in Table 1.

Examples of different marketed fluoroquinolone drugs used as broad-spectrum antimicrobials are illustrated in Fig. 1.

RESULTS AND DISCUSSION

Chemistry

The synthetic routes of the desired compounds illustrated in Schemes 1-3. Firstly, 6-bromo-4-hydroxyquinaldine, compound 1, was prepared according to the reported method (25), and was reacted with phosphorus pentasulfide in refluxing dry xylene to afford the thiol derivative 2. The latter compound 2 was coupled with a solution of the activated cyclic bromo sugar 2, 3, 4, 6-tetra-O-acetyl-α-D-gluco or galacto/pyranosyl bromide at room temperature, in potassium hydroxide solution to afford the corresponding thioglycoside derivatives 3a, b. Alternatively, refluxing of compound 1 with phosphorus oxychloride afforded 6-bromo-4-chloroquinaldine 4 (25), which in turn was converted to the corresponding hydrazine derivative 5 (35) via the reaction of compound 4 with hydrazine hydrate (98%) in refluxing ethanol. Then, product 5 was reacted with the activated cyclic bromo sugar 2, 3, 4, 6-tetra-O-acetyl-α-D-glucopyranosyl bromide in an acidic medium to afford the corresponding N-glycoside derivatives 6a, b. Additionally, condensation of compound 5 with furfural or

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Table 2. Minimum inhibitory concentration [MIC](µg/mL) of the tested compounds against selected microorganisms strains using agar dilution method.

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benzaldehyde in glacial acetic acid yielded the hydrazone derivatives 7a, b, respectively. Furthermore, the hydrazine derivative 5 was refluxed with methyl or phenyl isothiocyanate in dry benzene to give the corresponding thiosemicarbazide derivatives 8a, b (Scheme 1).

Also, conversion of the chloroquinaldine derivative 4 to 4-(p-acetylanilino) derivative 9 was proceeded according to the reported method (25). Compound 9 was condensed with thiosemicarbazide or phenyl thiosemicarbazide to afford the thiosemicarbazone derivatives 10a, b (25) respectively. Each of the derivatives 10a, b was reacted with phenacyl bromide to afford the thiazole products 11a, b. On the other hand, compounds 10a, b were reacted with ethyl bromoacetate in absolute ethanol containing few drops of piperidine to give the thiazolidinone derivatives 12a, b. Moreover, compound 9 was refluxed with semicarbazide hydrochloride in the presence of sodium acetate to give the semicarbazide derivative 13. Oxidative cyclization of compound 13 with thionyl chloride or selenium dioxide in glacial acetic acid afforded the corresponding thiadiazole or selenadiazole derivatives 14a, b, respectively (Scheme 2).

Also, the hydrazine derivative 5 was refluxed with acetyl chloride or benzoyl chloride, to give the hydrazide derivatives 15a, b, respectively, which underwent cyclocondensation by refluxing in acetic acid to give the corresponding cyclized imidazoquinoline derivatives 16a, b. Furthermore, compound 5 was reacted with 2-(ethoxymethylene) malononitrile in absolute ethanol to give the pyrazolo carbonitrile derivative 17 from which, when treated with oxalyl chloride in dry benzene, at room temperature, the pyrazolo pyrimidine derivative 18 was obtained (Scheme 3).

The structures of the new synthesized compounds were established and confirmed on the bases of their elemental analyses and spectral data (IR, 1H-NMR, 13C-NMR and MS.)

Biological evaluation

Some of the newly synthesized compounds were screened for their antibacterial activity using the agar dilution method (36). Ciprofloxacin and flucanazole were used as reference drugs. The results were recorded for each tested compound as the average diameter of inhibition zones of bacterial or fungal growth around the discs in mm. The minimum inhibitory concentration (MIC) measurement was determined for compounds that showed significant growth inhibition zones using the agar dilution method (36). The MIC (mg/mL) values of the active compounds against the tested bacterial and fungal strains are recorded in Table 2.

Each of B. subtilis and S. aureus were employed as Gram positive microorganisms, while, E. coli, K. pneumonia and P. aeruginosa were used as Gram negative bacteria. Also, C. albicans represented the fungi in the present investigation. The majority of the compounds showed an antibacterial effect towards Gram positive bacteria that can be described generally as moderate effect. Tables 1, 2 depicts the antibacterial effect of the tested compounds measured as zones of inhibition as well as minimal inhibitory concentrations. The hydrazine derivative of the present nucleus (compound 7a) is effective towards Gram positive bacteria (S. aureus and B. subtilis), some Gram negative (E. coli and P. aeruginosa) and C. albicans, a result that represents it as a promising broad spectrum antimicrobial. However, the hydrazide derivative (compound 15a) showed antibacterial effect only toward B. subtilis. The other compounds showed moderate effects against the tested Gram positive bacteria as presented in (Table 1). Also, the tested compounds showed variability in the MIC results. The results of the microbial sensitivity of the selected Gram negative microorganisms revealed that, each of the imidazo derivative 16a and the pyrazolo pyrimidine derivative 18 affected S. typhi with lower MIC in case of the derivative 16a (2 mg) about one half that of the

Figure 1. Some marketed quinolone drugs
derivative 18 (4 mg). Also, E. coli was sensitive to compound 16a with MIC of 1 mg. On the other hand, K. pneumoniae and P. aeruginosa were moderately sensitive to compounds 6a and 7a, respectively, in almost equipotent effect. In an attempt to investigate the antifungal effect of the newly synthesized compounds, it was found that only derivative 7a showed a moderate effect against C. albicans (4 mg). Figure 2 summarizes the effects of the tested compounds against Gram positive microorganisms B. subtilis and S. aureus. This figure shows that the compound 7a represented the lowest MIC (0.5, 0.25 mg/mL). In general, the MIC results of the tested compounds with respect to B. subtilis ranged from 0.5 to 4 mg/mL, while for S. aureus lies between 0.25 and 4 mg/mL.

Scheme 1. Synthetic pathways of compounds 2-8a, b
Synthesis and antimicrobial evaluation of some new quinaldine derivatives

The mass spectra were recorded on GCMC-QP 1000 EX Shimadzu Gas Chromatography MS spectrometer, Japan E.I. 70 ev. Elemental analysis (C, H, N) were carried out at the Micro Analytical Center, Faculty of Science, Cairo University, Egypt, and were in full agreement with the proposed structures within ± 0.2-0.3% of the theoretical values. All reagents were of commercial quality and were used.

**EXPERIMENTAL**

**General**

Melting points were obtained on a Barnstead 9001 Electrothermal melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer FT-IR Spectrum BX Spectrometer at cm⁻¹ scale using KBr discs. ¹H-NMR and ¹³C-NMR were recorded on a JEOL 300 MHz Spectrometer, Japan and chemical shift values were expressed in δ values (ppm) relative to tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. The mass spectra were recorded on GCMC-QP 1000 EX Shimadzu Gas Chromatography MS spectrometer, Japan E.I. 70 ev. Elemental analysis (C, H, N) were carried out at the Micro Analytical Center, Faculty of Science, Cairo University, Egypt, and were in full agreement with the proposed structures within ± 0.2-0.3% of the theoretical values. All reagents were of commercial quality and were used.

![Scheme 2. Synthetic pathways of compounds 9-14a, b](image-url)
without further purification. Reaction progress was monitored by analytical thin layer chromatography (TLC) on precoated (0.75 mm) silica gel GF254 plates (E. Merck, Germany) and the products were visualized by UV light.

Chemistry

6-Bromo-2-methylquinoline-4-thiol (2)

To a mixture of the quinaldine derivative 1 (0.01 M) in dry xylene (10 mL), (0.02 M) of phosphorus pentasulfide was added and heated under reflux for 6 h. After cooling, the solvent was evaporated under reduced pressure the solid formed was washed well with water, filtered off, and recrystallized from DMF/water.

Yield 65%; m.p.: 235°C; IR (\(\nu_{\text{max}}/\text{cm}^{-1}\)): 1610 (C=C); \(^1\)H-NMR (DMSO-\(d_2\), \(\delta\), ppm): 2.2 (s, 1H, SH), 2.3 (s, 3H, CH\(_3\)), 6.8 (s, 1H, H-3), 7.5-8.1 (m, 3H, Ar); \(^{13}\)C-NMR (DMSO-\(d_2\), \(\delta\), ppm): 23.9 (CH\(_3\)-quinaldine), 119.8, 120.6, 128.5, 129.1, 132.9, 136.4, 146.8, 158.9 (Ar-C); MS: m/z (%): 253, 255 (M\(^+\), M\(^+2\), 8.98, 8.7) consistent with the molecular formula (C\(_{10}\)H\(_8\)BrNS).

6-Bromo-2-methyl-4-(2',3',4',6'-tetra-O-acetyl-\(\beta\)-D-galactopyranosyl thio) quinoline (3a, b)

A mixture of compound 2 (0.01 M) in ethanol (10 mL) and a solution of aqueous potassium hydroxide (0.01 M) dissolved in distilled water (5 mL) was added to a solution of 2, 3, 4, 6-tetra-O-acetyl-\(\alpha\)-D-gluco- or galactopyranosyl bromide (0.01 M) in acetone (7 mL). The reaction mixture was stirred at room temperature for 5 h. After completion of the reaction, the mixture was concentrated under reduced pressure and poured onto ice-water. The solid formed was washed well with water and recrystallized from ethanol.

Yield 70%; m.p.: 140°C; IR (\(\nu_{\text{max}}/\text{cm}^{-1}\)): 1745 (C=O); \(^1\)H-NMR (DMSO-\(d_2\), \(\delta\), ppm): 1.94-2.2 (m, 12H, 4xOAc), 2.3 (s, 3H, CH\(_3\)), 3.9 (m, 2H, 6'-H\(_2\)), 4.1-5.1 (m, 5H, H-5', 4', 3', 2', 1'), 6.8 (s, 1H, H-3), 7.5-8.1 (m, 3H, Ar); \(^{13}\)C-NMR (DMSO-\(d_2\), \(\delta\), ppm): 21.08 (4 x COCH\(_3\)), 23.2 (CH\(_3\)-quinaldine) 61.2 (CH\(_2\), C-6'), 62.8, 63.9, 67.1, 70.8, 80.7 (C-5', 4', 3', 2', 1'), 119.8, 120.6, 128.5, 129.1, 130.2, 132.9, 143.4, 146.8, 158.9 (Ar-C and CN); 168.6 (4 x C=O); MS: m/z (%): 583, 585 (M\(^+\), M\(^+2\), 5.2, 5.0) consistent with the molecular formula (C\(_{24}\)H\(_{26}\)BrNO\(_9\)S).

6-Bromo-2-methyl-4-(2',3',4',6'-tetra-O-acetyl-\(\beta\)-D-glucopyranosyl thio) quinoline (3b)

Yield 65%; m.p.: 127°C; IR (\(\nu_{\text{max}}/\text{cm}^{-1}\)): 1744 (C=O); \(^1\)H-NMR (DMSO-\(d_2\), \(\delta\), ppm): 1.92-2.1 (m, 12H, 4 x OAc), 2.3 (s, 3H, CH\(_3\)), 4.01 (m, 2H, 6'-H\(_2\)), 4.02-5.09 (m, 5H, H-5', 4', 3', 2', 1'), 6.85 (s, 1H, H-3),

![Scheme 3. Synthetic pathways of compounds 15-18a, b](image-url)
H-3), 7.5-8.1 (m, 3H, Ar); 3'-NMR (DMSO-d$_6$, δ, ppm): 21.08 (4 x COCH$_3$), 22.91 (CH$_3$-quininalde), 60.91 (CH$_2$, C-6'), 61.3, 62.1, 65.1, 68.7, 80.3 (C-5', 4', 3', 2', 1'), 119.8, 120.6, 128.5, 129.1, 130.2, 132.9, 143.4, 146.8, 158.9 (Ar-C and C=N); 168.3 (4 x C=O); MS: m/z (%): 583, 585 (M$^+$, M$^{+2}$, 3.6, 2.9) consistent with the molecular formula (C$_{24}$H$_{26}$Br N$_2$O$_9$).

1-(6-Bromo-2-methylquinolin-4-yl)-2-(2',3',4',6'-tetra-O-acetyl-β-D-galacto and glucopyranosyl- yl) hydrazine (6a, b)

To a solution of compound 5 (0.01 M) in acetone (10 mL) 2, 3, 4, 6-tetra-O-acetyl-α-D-glucose or galactopyranosyl bromide (0.01 M) in acetone (15 mL) was added followed by addition of few drops of acetic acid, the reaction mixture was heated with stirring on a water bath at 55-65°C for 10 h. After the reaction was completed, the solvent was concentrated under reduced pressure. After cooling, the separated solid was collected by filtration, washed with water, dried and recrystallized from ethanol.

1-(6-Bromo-2-methylquinolin-4-yl)-2-(2',3',4',6'-tetra-O-acetyl-β-D-galacto) hydrazine (6a)

Yield 72%; m.p.: 290°C; IR (c$_{\text{vap}}$/cm$^{-1}$): 3380 (br, NH), 1740 (C=O), 1317 (C-N); 1H-NMR (DMSO-d$_6$, δ, ppm): 1.97-2.02 (m, 12H, 4 x OAc), 2.31 (s, 3H, CH$_3$), 3.91 (s, 2H, 6-H$_2$), 4.02-5.50 (m, 5H, H-5', 4', 3', 2', 1'), 6.3 (s, 1H, Ar, H-3), 7.6-8.1 (m, 3H, Ar), 9.8 (s, 1H, 1NH), 10.5 (s, 1H, 2NH); 13C-NMR (DMSO-d$_6$, δ, ppm): 21.85 (4 x OAc), 22.91 (CH$_3$-quininalde), 60.91 (CH$_2$, C-6'), 61.93, 62.32, 64.34, 68.94, 71.63, (C-5', 4', 3', 2', 1'), 110.71, 111.62, 117.6, 120.6, 129.2, 143.6, 144.6, 147.49, 158.5 (Ar-C, C=N); MS: m/z (%): 329, 331 (M$^+$, M$^{+2}$, 26.2, 23.8) consistent with the molecular formula (C$_{24}$H$_{26}$Br N$_2$O$_9$).

1-(6-Bromo-2-methylquinolin-4-yl)-2-(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl) hydrazine (6b)

Yield 70%; m.p.: 230°C; IR (c$_{\text{vap}}$/cm$^{-1}$): 3380 (br, NH), 1714 (C=O), 1317 (C-N); 1H-NMR (DMSO-d$_6$, δ, ppm): 1.97-2.02 (m, 12H, 4 x OAc), 2.31 (s, 3H, CH$_3$), 3.91 (s, 2H, 6-H$_2$), 4.02-5.50 (m, 5H, H-5', 4', 3', 2', 1'), 6.3 (s, 1H, Ar, H-3), 7.6-7.9 (m, 8H, 7 Ar-H and N=CH), 8.1 (s, 1H, Ar, H-5) 9.3 (s, 1H, NH); 13C-NMR (CDCl$_3$, δ, ppm): 35.0) consistent with the molecular formula (C$_{17}$H$_{14}$Br N$_3$).
(CDCl₃, δ, ppm): 24.2 (quinaldine-CH₃), 28.2 (NCH₃), 100.71, 116.92, 117.6, 120.6, 129.2, 132.4, 146.6, 147.49, 158.5 (Ar-C, C=N), 175.9 (C=O), MS; m/z (%): 324, 326 (M⁺, M⁺2, 22.8, 21.5) consistent with the molecular formula (C₂₁ H₁₈ Br N₅ O S).

1-(6-Bromo-2-methylquinolin-4-yl)-4-phenylthiosemicarbazide (8b)

Yield: 85%, m.p.: 300°C; IR (νmax/cm⁻¹): 3290, 3216 (3NH), 1623 (C=O); 1H-NMR (CDCl₃, δ, ppm): 2.3 (s, 3H, quinaldine-CH₃), 6.5 (s, 1H, H-3), 6.7 (d, J = 8.7, 2H, Ar, H-2,6'), 7.6-8.1 (m, 6H, Ar), 8.3 (s, 1H, NH), 9.8 (s, 1H, NH), 10.1 (s, 1H, NH); 13C-NMR (CDCl₃, δ, ppm): 24.2 (quinaldine-CH₃), 110.71, 116.92, 117.6, 120.6, 123.6, 126.9, 128.7, 129.2, 132.4, 136.8, 146.6, 147.49, 158.5 (Ar-C, C=N); 175.5 (C=O), MS; m/z (%): 386, 388 (M⁺, M⁺2, 18.3, 18.0) consistent with the molecular formula (C₁₇ H₁₅ Br N₄ S).

1-[6-Bromo-2-methylquinolin-4-yl]-4-ethyldienebenzenamine-1-hydrazono]-3-phenylthiazolidin-4-one (12b)

Yield: 77%, m.p.: 210°C; IR (νmax/cm⁻¹): br. 3321 (NH), 1623 (C=O), 1635 (C=O); 1H-NMR (CDCl₃, δ, ppm): 2.02 (s, 3H, CH₃), 2.3 (s, 3H, quinaldine-CH₃), 3.9 (s, 2H, thiazolidinone), 6.2 (s, 1H, H-3), 7.0 (d, J = 7.8, 2H, Ar, H-2,6'), 7.6-7.9 (m, 4H, Ar-H), 8.1 (s, 1H, NH), 9.5 (s, 1H, NH), 9.8 (s, 1H, NH); 13C-NMR (CDCl₃, δ, ppm): 19.3 (CH₃), 24.2 (quinaldine-CH₃), 107.3, 109.5, 116.01, 116.04, 118.1, 120.8, 126.01, 127.5, 127.9, 129.3, 129.5, 132.6, 143.02, 144.7, 147.3, 156.4, 157.6, 157.9, 159.02 (Ar-C, C=N); MS; m/z (%): 467, 469 (M⁺, M⁺2, 16.8, 16.8) consistent with the molecular formula (C₃₃ H₂₆ Br N₅ S).
Synthesis and antimicrobial evaluation of some new quinoline derivatives

1-(6-Bromo-2-methylquinolin-4-ylamino)-4-phenylethlylidene] semicarbazide (13)

A mixture of semicarbazide hydrochloride (0.01 M), and crystalline sodium acetate in water (10 mL) was added while stirring to a solution of compound 9 (0.001 M) in ethanol (20 mL). Stirring was continued for 15 min, at 5°C, the solid product was filtered off, washed with water, dried and recrystallized from ethanol.

Yield: 90%, m.p.: 275°C; IR (ν<sub>max</sub>/cm<sup>-1</sup>): 3413 (NH₂), 3276 (br, 2NH), 1680 (C=O); ¹H-NMR (CDCl₃, δ, ppm): 2.1 (s, 3H, CH₃), 2.3 (s, 3H, quinaldine-CH₃), 6.07 (s, 1H, NH₂), 6.3 (s, 1H, H-3), 6.6 (d, J = 8.7, 2H, H-2’, 6’), 6.9-8.1 (m, 5H, Ar-H), 9.04 (s, 1H, NH), 10.2 (s, 1H,NH); ¹³C-NMR (CDCl₃, δ, ppm): 22.5 (CH₃), 24.2 (quinaldine-CH₃), 107.3, 116.01, 116.4, 118.1, 120.8, 123.6, 129.9, 132.6, 144.5, 146.7, 147.3, 157.9, 160.2, 161.7, 162.1 (Ar-C, C≡N and C=O), MS; m/z (%): 411, 413 (M+, M+2, 24.5, 23.0) consistent with the molecular formula (C₁₉H₁₈BrN₅O).

4-(6-Bromo-2-methylquinolin-4-yl)-(1, 2, 3-thiadiazol-4-yl] benzenamine (14a)

To a stirred solution of the semicarbazide 13 (0.001 M) in acetic acid (2 mL), at zero °C, thionyl chloride (0.025 M) was added dropwise and the resulting mixture was further stirred for 30 min at this temperature, then, the reaction mixture was left at room temperature overnight, and a saturated solution of sodium bicarbonate was added. The product was extracted with chloroform (40 mL), the organic layer was washed well with water, dried with anhydrous sodium sulfate. After evaporation of the solvent under reduced pressure, the solid formed was filtered off, dried and recrystallized from DMF affording the title compound.

Yield: 89%, m.p.: > 300°C; IR (ν<sub>max</sub>/cm<sup>-1</sup>): 3313, (NH), 1615(C=N); ¹H-NMR (CDCl₃, δ, ppm): 2.3 (s, 3H, CH₃), 5.9 (s, 1H, selenadiazole-H), 6.2 (s, 1H, H-3), 6.9-8.1 (m, 7H, Ar-H), 10.5 (s, 1H, NH); ¹³C-NMR (CDCl₃, δ, ppm): 24.2 (CH₃), 107.3, 111.5, 116.01, 116.4, 118.1, 120.8, 123.6, 126.9, 129.3, 132.6, 144.5, 145.6, 147.3, 159.2, 157.9 (Ar-C,C≡N); MS: m/z (%): 443, 445 (M⁺; M²⁺, 50.6, 21.4) consistent with the molecular formula (C₁₈H₁₃BrN₄S).

1-(6-Bromo-2-methylquinolin-4-yl)-acetohydrazide (15a), 1-(6-bromo-2-methylquinolin-4-yl)-benzohydrazide (15b)

A mixture of compound 5 (0.01 M) and the suitable acid chloride, namely acetyl chloride or benzoyl chloride (5 mL) was refluxed for 3 h. Then, the excess acid chloride was evaporated under reduced pressure and the solid product was washed with cold water, collected by filtration and recrystallized from ethanol.

Yield: 73%, m.p.:> 300°C; IR (ν<sub>max</sub>/cm<sup>-1</sup>): br. 3288 (NH), 1663 (C=O), 1591 (C=N); ¹H-NMR (CDCl₃, δ, ppm): 2.1 (s, 3H, COCH₃), 2.3 (s, 3H, quinaldine-CH₃), 6.2 (s, 1H, H-3), 7.6-8.1 (m, 3H, Ar-H); ¹³C-NMR (CDCl₃, δ, ppm): 24.2 (quinaldine-CH₃), 24.6 (CH₃), 107.3, 116.4, 118.1, 120.8, 123.6, 129.9, 132.6, 144.5, 147.3, 157.9, 160.2, 161.7, 162.1 (Ar-C, C≡N and C=O), MS: m/z (%): 293, 295 (M⁺; M²⁺, 10.7, 7.8) consistent with the molecular formula (C₁₂H₁₂BrN₃O).

1-(6-Bromo-2-methylquinolin-4-yl)-acetoxydrazide (15a)

Yield: 76%, m.p.:> 300°C; IR (ν<sub>max</sub>/cm<sup>-1</sup>): br. 3310 (NH) 1700 (C=O), 1370 (C≡N); ¹H-NMR (CDCl₃, δ, ppm): 2.3 (s, 3H, quinaldine-CH₃), 6.3 (s, 1H, H-3), 7.5-8.2 (m, 6H, Ar-H); ¹³C-NMR (CDCl₃, δ, ppm): 24.2 (quinaldine-CH₃), 24.6 (CH₃), 107.3, 116.4, 118.1, 120.8, 123.6, 146.7, 147.3, 158.02 (Ar-C,C≡N), 165.1 (C=O), MS: m/z (%): 293, 295 (M⁺; M²⁺, 10.7, 7.8) consistent with the molecular formula (C₁₈H₁₃BrN₄O).

1-(6-Bromo-2-methylquinolin-4-yl)-benzohydrazide (15b)

Yield: 73%, m.p.:> 300°C; IR (ν<sub>max</sub>/cm<sup>-1</sup>): 3310 (NH), 1643 (C=O), 1591 (C=N); ¹H-NMR (CDCl₃, δ, ppm): 2.1 (s, 3H, COCH₃), 2.3 (s, 3H, quinaldine-CH₃), 6.2 (s, 1H, H-3), 7.6-8.1 (m, 3H, Ar-H); ¹³C-NMR (CDCl₃, δ, ppm): 24.2 (quinaldine-CH₃), 24.6 (CH₃), 107.3, 116.4, 118.1, 120.8, 123.6, 146.7, 147.3, 158.02 (Ar-C,C≡N), 165.1 (C=O), MS: m/z (%): 293, 295 (M⁺; M²⁺, 10.7, 7.8) consistent with the molecular formula (C₁₈H₁₃BrN₄O).
8-Bromo-2,4-dimethyl-1H-imidazo[4,5-c]quinoline (16a), 8-bromo-4-methyl-2-phenyl-1H-imidazo[4,5-c]quinoline (16b)

A mixture of compounds 15a, b (0.01 M) and acetic acid (25 mL) was refluxed for 3 h. The reaction mixture was evaporated under reduced pressure, after cooling, the reaction mixture was poured onto ice-water and the solid formed was washed with water during filtration, dried and recrystallized from ethanol.

8-Bromo-2,4-dimethyl-1H-imidazo[4,5-c]quinoline (16a)

Yield: 69%, m.p.: 195°C; IR (ν max/cm⁻¹): 3231 (NH), 1591 (C=N). 1H-NMR (CDCl₃, δ, ppm): 2.2 (s, 3H, CH₃), 2.4 (s, 3H, quinaldine-CH₃), 7.6-8.1 (m, 3H, Ar), 9.2 (s, 1H, NH) 13C-NMR (CDCl₃, δ, ppm): 19.1(CH₃), 21.2 (quinaldine-CH₃), 119.6, 120.9, 121.9, 121.7, 127.6, 129.01, 132.8, 142.6, 145.8, 158.9 (Ar-C, C=N); MS; m/z (%): 275, 277 (M⁺, M+2, 50.7, 21.9) consistent with the molecular formula (C₁₂H₁₀BrN₃).

8-Bromo-4-methyl-2-phenyl-1H-imidazo[4,5-c]quinolone (16b)

Yield: 70%, m.p.: 154°C; IR (ν max/cm⁻¹): 3316 (NH), 1627 (C=N). 1H-NMR (CDCl₃, δ, ppm): 2.4 (s, 3H, quinaldine-CH₃), 7.1-7.9 (m, 7H, Ar), 8.1 (s, 1H, Ar-H), 9.3 (s, 1H, NH); 13C-NMR (CDCl₃, δ, ppm): 21.2 (CH₂), 119.6, 120.9, 121.9, 121.7, 126.1, 127.6, 127.9, 128.04, 128.9, 132.8, 142.6, 145.8, 158.9 (Ar-C, C=N); MS; m/z (%): 337, 339 (M⁺, M+2, 5.5, 5.0) consistent with the molecular formula (C₁₇H₁₂BrN₃).

5-Amino-1-(6-bromo-2-methylquinolin-4-yl)-1H-pyrazole-4-carbonitrile (17)

To a solution of compound 5 (0.01 M) in absolute ethanol (15 mL), 2-(ethoxymethylene) malononitrile (0.02 M) was added, the reaction mixture was heated under reflux for 3 h., the excess solvent was evaporated under reduced pressure, after cooling, the solid formed was filtered off, dried and recrystallized from ethanol.

Yield: 90 %, m.p.: > 300°C; IR (ν max/cm⁻¹): 3412 (NH₂), 2219 (CN); 1H-NMR (CDCl₃, δ, ppm): 2.3 (s, 3H, CH₃), 4.01 (s, 1H, NH₂), 6.9 (s, 1H, H-3), 7.3 (s, 1H, pyrazole), 7.5-8.1 (m, 3H, Ar ); 13C-NMR (CDCl₃, δ, ppm): 24.1 (CH₂), 91.5, 111.7, 115.9, 119.6, 125.8, 127.1, 127.9, 130.4, 140.5, 142.7, 143.1, 147.9, 157.1(Ar-C, C=N); MS; m/z (%): 327, 329 (M⁺, M+2, 31.8, 28.4) consistent with the molecular formula (C₁₄H₁₀BrN₅).

1-(6-Bromo-2-methylquinolin-4-yl)-4,5-dihydro-4-oxo-1H-pyrazolo[3,4-d]pyrimidine-6-carbon-yl chloride (18)

To a mixture of compound 17 (0.01 M) in dry benzene (15 mL) oxalyl chloride (0.015 M) was added. The mixture was stirred at room temperature for 24 h., the solid product was collected by filtration, washed well with water, dried and recrystallized from ethanol.

Yield: 88%, m.p.: 288°C; IR (ν max/cm⁻¹): 3215 (NH), 1723, 1675 (2C=O); 1H-NMR (CDCl₃, δ, ppm): 2.3 (s, 3H, Ar-CH₃), 6.9 (s, 1H, H-3), 7.5-8.1 (m, 4H, Ar and 1H, pyrazole), 8.9 (s, 1H, NH), 13C-NMR (CDCl₃, δ, ppm): 24.1(CH₂), 106.3, 111.7, 112.9, 119.6, 127.1, 127.9, 130.4, 132.5, 140.9, 146.7, 147.9, 158.1, 159.9 (Ar-C, C=N); MS; m/z (%): 417, 419 (M⁺, M+2, 4.4, 6.1) consistent with the molecular formula (C₁₆H₉BrClN₅O₂).

Antimicrobial evaluation

Some of the synthesized new compounds were investigated for their antimicrobial effect toward each of Gram positive as well as Gram negative bacteria and fungi. Strains used to detect the antimicrobial activity of the prepared compounds included, Staphylococcus aureus ATCC 29213 and Bacillus subtilis ATCC 3366 as Gram positive, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Salmonella typhi ATCC 25566 and Klepsiella pneumoniae ATCC 13883 as Gram negative, as well as Candida albicans NRRL Y-477 to present fungus.

The agar dilution method was used to study the microbiological inhibition of the prepared compounds. The Mueller–Hinton agar plates were prepared and adjusted to contain serial twofold dilutions of the tested compounds in-house. Each plate was inoculated with a specific microorganism at 0.5 McFarland and incubated at 37°C 24 h for.

The MIC was considered the lowest concentration of an antimicrobial agent that completely inhibited growth on the agar as detected visually. Ciprofloxacin and fluconazole were used as reference drugs.

CONCLUSION

The most common powerful quinoline derivatives as antibiotics are fluoroquinolones. However, literature survey reported that most of them have adverse effects which may occur almost anywhere in the body. Depending upon the above knowledge and in continuation to our previous efforts dealing
with the synthesis of various 6-bromoquinaldine derivatives of strong antimicrobial potency, a novel series of bromoquinaldine derivatives incorporated into or fused to various biologically active heterocyclic ring systems or glycosides were synthesized and evaluating some of them as antibacterial and antifungal. All the tested compounds exhibited potential antibacterial activity against Gram-positive bacteria except compound 6a which interns appeared to have activity against the Gram negative K. pneumoniae. From that we can conclude that substituted quinaldine compounds may provide more activity by further study trying to modify their substitutions to give new derivatives with expected improved antimicrobial activity and that may be the subject of our future work.

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A cream is a heterogeneous formulation that is prepared by mixing two liquids that cannot be mixed without the help of a third ingredient known as emulsifying agent. One out of these two liquids serves as the dispersion medium while the other is distributed throughout the dispersion medium in the form of droplets. The range of these droplets in the creams used for pharmaceutical purpose is about 0.1 to 50 µm (1). An oil-in-water (o/w) cream is one in which oil is present as droplets in the dispersion medium that is water while the reverse is true for water-in-oil (w/o) emulsion. When a small amount of oil is required like in shaving or conditioning creams, o/w-emulsions are used while the w/o emulsions are desired where more oily preparation is required like emollient creams and sunscreens. There is a number of emulsifiers that are used to formulate the emulsions but these may cause irritancy and sensitivity to the user. So it is necessary to develop the formulation using emulsifying agents that do not cause such adverse reactions. Nonionic surfactants as emulsifiers are considered safe with regard to such adverse reactions (2).

Various processes can account for the instability of the emulsions such as creaming, Ostwald ripening, phase separation, rupture and coalescence (3). Physical and chemical stability of the formulations can be assessed promptly by keeping them at different temperatures for some time. By rheological measurement one can estimate not only the physical stability but also the quality, effectiveness and purpose of system (4).

Bombax ceiba belongs to the family Bombacaceae and commonly known as silk cotton tree. It is a remarkable medicinal plant of Indian tropical and subtropical regions including Pakistan. In old-style medicine systems that are still in practice in India like Ayurveda, Siddha and Unani, it is used to cure many diseases like sexual incapacity, vaginal infections and to stop bleeding from wounds (5). Many diseases can be cured by using different parts of B. ceiba. Stem bark acts as acrid, demulcent,
diuretic, anti-inflammatory, slightly astringent and tonic. It can be used for facial illnesses such as marks, acne vulgaris, pigmentation disorder, inflammation, blister and burning sensation. Stem bark contains lupeol, β-sitosterol, shamimicin and apigenin (6).

The aim of the present study was to prepare and investigate physical stability of the cream containing *B. ceiba* extract that could serve as a cosmetic product and contribute to the skin care. The stability of the formulation was evaluated using different parameters by placing at different storage conditions over time.

**EXPERIMENTAL**

**Plant material identification**

*Bombax ceiba* bark was collected locally from Bahawalpur, Pakistan and validated by the Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur, Pakistan. A voucher specimen (No. 3524/CIDS/IUB) was issued after depositing the sample in the herbarium of CIDS, The Islamia University of Bahawalpur, Pakistan. ABIL EM 90 or cetyl PEG/PPG-10/1 dimethicone, was obtained from the Franken (Germany) and paraffin oil from Merck (Germany).

**Extraction and cream preparation**

*Bombax ceiba* stem bark was collected locally and extracted with 80% aqueous-methanol. Previously weighed contents of oily phase including paraffin oil (14%) and emulsifier (ABIL-EM 90) (4.5%) were heated up to 75 ± 1°C. Aqueous phase comprising of water (77.5%) and *B. ceiba* extract (4%) was also heated to the same temperature (75 ± 1°C). Subsequently, aqueous phase was added to the oil phase with constant stirring with the help of a stirrer (Euro-Star, IKA D 230, Germany) at a speed of 2000 rpm for 10 min till the complete addition of aqueous phase. 2 to 3 drops of rose oil as fragrant were added during stirring. Mixer speed was adjusted to 1000 rpm after the aqueous phase was added and continued for almost 10 min to homogenize the ingredients. This process of emulsification was further carried on with reduction in speed (500 rpm) for complete homogenization until the emulsion was cooled down to room temperature.

**Characterization of the cream**

Examination was done by keeping the samples of the cream at four different conditions of storage i.e., 8°C, 25°C, 40°C and 40°C + 75% RH (relative humidity) for 12 weeks to assess the stability under these conditions. The cream was evaluated physically (color, liquefaction and phase separation) under these conditions. Centrifugation (Centrifuge Machine, Hettich EBA 20 Germany) was carried out at a speed of 5000 rpm and at 25°C for 30 min by employing the sample (few grams) in a disposable centrifugal tube. Rheological parameters for the formulations were determined at 25°C after preparation and at different time intervals for 12 weeks, taking 0.5 g of the sample. Rheology was determined using

---

**Table 1. Variation in physical characteristics of the formulation at various time intervals stored at 8°C, 25°C, 40°C and 40°C/75% RH.**

<table>
<thead>
<tr>
<th>Physical characteristic</th>
<th>Storage temperature</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 Week</td>
</tr>
<tr>
<td>Liquefaction</td>
<td>8°C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40°C/75% RH</td>
<td>-</td>
</tr>
<tr>
<td>Color</td>
<td>8°C</td>
<td>LP</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>LP</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>LP</td>
</tr>
<tr>
<td></td>
<td>40°C/75% RH</td>
<td>LP</td>
</tr>
<tr>
<td>Phase separation</td>
<td>8°C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>40°C/75% RH</td>
<td>-</td>
</tr>
</tbody>
</table>

- = No change; + = slight change; LP = light pink.
a CP 41 spindle of a cone-plate rheometer (Brookfield DV-III Ultra). Values for consistency index (related to the system viscosity) and flow index (related to the system pseudo plasticity) were obtained by the power law. Increased shear stresses were applied on the samples and change in viscosity was observed.

**Power law**

The power law equation is as shown in Eq. 1.

\[
\tau = kD^n
\]

where \( \tau \) = shear stress, \( D \) = yield stress (stress at zero shear rate), \( k \) = plastic viscosity, and \( n \) = shear rate

The calculated parameters for this model are flow index (no units), consistency index (cP) and confidence of fit (%).

**Microscopic examination**

Stability of the creams can be established by an essential characteristic that is the droplet size of internal phase. With the help of an optical microscope (Eclipse E200, Nikon, Japan), to which a CCD camera was attached the droplet size was determined. The images were processed using mini see software (V. 1.1). Firstly, a minute quantity of sample stored at different temperatures was taken on the slide then it was diluted with the continuous phase and covered with the cover slip. After that it was observed under the microscope by the 100× lens. The droplet diameter was determined by stage micrometer and graticule. The micrographs obtained are shown (Fig. 2).

**RESULTS**

The creams were examined on a physical (color, phase separation and liquefaction) basis and results are shown in Table 1. It was noted that no change in the color of the formulation occurred. There was no phase separation and liquefaction found except to slight phase separation and liquefaction at 40°C on the 90th day of study was found. In the present study, centrifugation test of freshly prepared sample and samples kept at different storage conditions was done. These evaluations were carried out for a period of 12 weeks at different storage conditions (8°C, 25°C, 40°C, and 40°C + 75% RH) and at specific time intervals.

We also evaluated the influence of storage conditions on the viscosity of the preparation by subjecting the formulations to the rheometer. Figure 1 presents the rheograms of the formulations stored at different temperatures. Measurements were recorded for the flow index and consistency index for the formulations and are shown in Table 2.

The droplet size is also an important characteristic of the emulsion so it was measured according to the procedure stated earlier. Figure 2 reveals the microscopic images of the cream containing B. ceiba extract. It was found that the droplet size was not only increased with time but this increase was also more prominent at higher temperature.

**DISCUSSION**

Storage at various temperatures is a well-known method to explore the stability of an emulsion. This method is easy to carry out because breaking of the emulsions is accelerated by thermal stress (7). The freshly prepared formulation was light pink in color and sustained the color throughout the study period (12 weeks). This resistance in change of color revealed the stability of the formulation at different storage conditions i.e., 8°C, 25°C, 40°C, and 40°C + 75% RH throughout the period of investigation. Phytochemical studies on various parts of B. ceiba exposed that it is rich in phenolic compounds (8). These phenolic compounds help retain the original color of the formulations by inhibiting the growth of microbes (9).

Flow characteristic of the cream is indicated by its viscosity and stability of emulsion can be assessed by determining the time and temperature effect upon viscosity (10). All samples kept at 8°C, 25°C and 40°C + 75% RH were stable and no liquefaction was observed.

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Table 2. Flow index and consistency index values determined at various time intervals after storage at 8°C, 25°C, 40°C and 40°C/75% RH.

<table>
<thead>
<tr>
<th>Time</th>
<th>8°C FI</th>
<th>25°C CI</th>
<th>40°C CF</th>
<th>40°C/75% RH CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Week</td>
<td>0.58</td>
<td>1485</td>
<td>99.6</td>
<td>0.58</td>
</tr>
<tr>
<td>4 Week</td>
<td>0.57</td>
<td>1125</td>
<td>99.8</td>
<td>0.51</td>
</tr>
<tr>
<td>8 Week</td>
<td>0.55</td>
<td>1045</td>
<td>99.7</td>
<td>0.61</td>
</tr>
<tr>
<td>12 Week</td>
<td>0.50</td>
<td>985</td>
<td>99.3</td>
<td>0.56</td>
</tr>
</tbody>
</table>

FI = Flow index; CI = Consistency index; CF = Confidence of fit (%)
observed. Very little liquefaction was seen in the sample kept at 40°C on 90th day. With the passage of time it is likely that some time and temperature driven processes reduce the viscosity of preparation leading to liquefaction (11). The droplets of internal phase move upward or downward causing creaming or sedimentation due to the density dissimilarity of the two phases (12). Slight phase separation was detected in the sample placed at 40°C on 90th day of observation. During creaming/sedimentation the droplets tend to grow leading to decrease in the total interfacial energy. This shift to a larger mean droplet size due to the coalescence of smaller droplets causes separation of the phases in some cases (12) while the emulsion remains stable at low temperatures because viscosity did not change (13).

Type of the emulsion can be determined by using different methods. We used electrical conductivity test for this purpose. Emulsion with water as an external phase can conduct the electricity because electricity can pass through the water while oil is a poor conductor of the electric current so when oil is the dispersion medium there will be no electrical conductivity (14). No electrical conductivity was found in the formulation under test so we can say that it was water in oil emulsion.

Physical stability of the creams can be evaluated by using the techniques such as centrifugation that can enhance the instability in the formulations (7). It was noted that after centrifugation there was no phase separation found in the samples kept at 8°C, 25°C and 40°C + 75% RH till the end of study. The appropriate speed of the mixer during the process of emulsification can prevent the formulation from breakage under accelerated studies (10). However, the sample exposed to 40°C exhibited phase separation after 60 days in the centrifugation test. By applying the centrifugal force creaming/sedimentation can be augmented due to change in the droplet size, structure and distribution (12).

Figure 1. Rheograms of formulation at various time intervals A) Formulation stored at 8°C, B) Formulation stored at 25°C, C) Formulation stored at 40°C, D) Formulation stored at 40°C/75% RH

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**Figure 1.** Rheograms of formulation at various time intervals A) Formulation stored at 8°C, B) Formulation stored at 25°C, C) Formulation stored at 40°C, D) Formulation stored at 40°C/75% RH
Right consistency (rheology) for a good sensation of the skin and better spreading is desired standard for the emulsions used as personal care products. Rheology can be used to display the information about the stability of the emulsion (15). In the current study it was observed that the formulations depicted shear thinning effect because viscosity of the formulations decreased with the increase in shear stress as can be incurred from the values of the flow index that are less than 1. It is the property that is very much required for good application and good feel of the formulation. It is necessary to expose the formulation to different temperatures and obtain the rheological data to get the valuable information about the product stability and consistency (15). The slight decrease in the consistency index was found in the formulations. Consistency index represents the viscosity of the formulations and it is known that consistency index normally decreases upon storage (4).

Physicochemical characteristics including the droplet size and rheological characteristics can be used to assess the emulsion stability. The droplet size of the disperse systems like emulsions can be achieved by using numerous methods (16). In the current investigation micrographs indicated that spherical globules of 2-4 µm were present in the freshly prepared formulations. These values are within the given range as mentioned in the literature for the pharmaceutical emulsions (1). After keeping the formulations on different storage conditions it was seen that the droplet size increased differently at various temperatures. The droplet size was increased up to 10.4 µm and 12.2 µm, respectively, at 8°C and 25°C after 12 weeks of storage. While the droplet size of the formulations kept at 40°C and 40°C/75% RH were found to be 17 µm and 25 µm after 12 weeks. It can be seen that at higher temperature the droplet size of the formulation was increased more as compared to the formulations kept at lower temperature. There is some evidence that the stability of the emulsion at lower temperatures may be because of the stable film of emulsifier around the droplets that allows less contact of globule during storage. Slight decrease in the viscosity with time at higher temperatures, 40°C and 40°C/75% RH allows more probability of the droplet size increase by coalescence (17).

CONCLUSION

From the current study, we can conclude that the cream containing Bombax ceiba stem bark extract in concentration of 4% exhibited promising stability and physicochemical characteristics at different storage conditions. This formulation showed no change in color or any liquefaction however a slight phase separation was seen at 40°C. The stabil-
ity of the cream can be increased by storing at low temperatures while higher temperature can affect it negatively. The formulation can offer a good delivery system for skin rejuvenation agents. Further in vivo studies have to be done to discover the benefits of the cream as a phytocosmetic agent.

Acknowledgment

The authors thank to the Chairman and Dean of the Faculty of Pharmacy & Alternative Medicine, The Islamia University of Bahawalpur, Pakistan for providing the lab facilities to conduct the study and the moral support given to them.

Conflict of interest

There is no conflict of interest associated with this work.

REFERENCE


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**EVALUATION OF ANTIBACTERIAL AND CARBONIC ANHYDRATE INHIBITORY POTENTIAL OF METHANOLIC EXTRACT OF NARDOSTACHYS JATAMANSI (D. DON) DC RHIZOMES**

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¹University College of Conventional Medicine, ²Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Bahawalpur, Pakistan ³Middle East Technical University, Ankara, Turkey

**Abstract:** Many antimicrobial drugs are going to become resistant to different pathogens, so the discovery of new antimicrobial products is an important public health concern. *Nardostachys jatamansi* (D. Don) DC (Valerianaceae) is an important traditional herbal medicine used as tranquillizer, CNS sedative, antiepilepsy, cardiotonic, diuretic, heptatonic, analgesic and in boils, itch and eye diseases. Carbonic anhydrase inhibitors are the potential source of treatment in case of glaucoma, hypertension and epilepsy and also used as diuretics. The aim of present study was to evaluate the antibacterial activity of methanolic extract of *N. jatamansi* against different bacterial strains and to estimate its carbonic anhydrase inhibitory potential. Antibacterial activity was evaluated by agar well diffusion assay and broth microdilution assay. The study revealed that *N. jatamansi* extract is sensitive to all tested bacterial strains. The zones of inhibition and MIC ranged from 8-22 at a concentration of 1-5 mg/mL and 0.3-0.6 mg/mL, respectively. Methanolic extract of *N. jatamansi* showed marked inhibition of carbonic anhydrase (IC₅₀ 712.41 ± 0.001 µg/mL) when compared with standard acetazolamide.

The results of the study suggest that *N. jatamansi* may be a valuable plant source of medicinally useful active compounds that can be helpful in bacterial infections and showed some relation with the traditional use of this plant in various diseases.

**Keywords:** *Nardostachys jatamansi*, antibacterial, carbonic anhydrase inhibitor

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*Nardostachys jatamansi* (D. Don) DC; belongs to Valerianaceae family. It has an important place in traditional medicine in the Indian subcontinent and the Middle East, being used mainly as a tranquilizer and CNS sedative. Moreover, it is also used for gastrointestinal hyperactivity (1). The roots of *Nardostachys jatamansi* are used traditionally in the treatment of convulsive ailments, epilepsy, hysteria, heart palpitations, boils, diseases of the eyes, itch, etc. (2). In the Unani system of medicine, Sunbul-ul-tib (*Nardostachys jatamansi*) has been mentioned as a heptatonic, cardiotonic, diuretic and analgesic (3).

For the medicinal purpose, rhizomes of *N. jatamansi* are mostly used. Macroscopically, the rhizome of the plant is cylindrical and elongated in shape, and fine fibers in a network cover it. Phytochemical analysis showed the presence of alkaloids, amino acids, sugars and tannins in hot and cold methanolic extracts (4). Valeranone sesquiterpenes are the principal active constituents in *N. jatamansi* oil and it causes sleep induction (1). It contains many other sesquiterpenes including jatamansinone, jatamansinolin, oroseolol, oroselone, seselin, jatamol A and B, valeranal, nardostachyin, nardosinone, spirojatamol, jatamansic acid, nardostachone, calarenol, coumarin, jatamansin, xanthogalin, seychelane and seychellene (5).

Six Gram negative (*Shigella dysenteriae*, *Corynebacterium striatum*, *Proteus vulgaris* *Escherichia coli*, *Pseudomonas aeruginosa*, *Kleb-
siella pneumoniae) and two Gram positive bacteria (Bacillus subtilis, Staphylococcus aureus) were included in the study. These bacterial strains were selected as these are the commonly infection causing bacteria in humans as Bacillus subtilis involve in the various allergic conditions of respiratory track, food poisoning and eye infections (6). Staphylococcus aureus is responsible for a variety of diseases including skin (boils, itch), soft tissue, bone, joint, food poisoning, cardiovascular, respiratory and wound infections. Pseudomonas aeruginosa can cause infections such as urinary tract infections, pneumonia, ear and eye infections and traumatic wound infections. Klebsiella pneumoniae causes urinary tract infections, wound infections, cholecystitis, meningitis and endocarditis. Escherichia coli is an opportunistic organism causing pneumonia and sepsis in immunocompromised host and meningitis (7).

Carbonic anhydrase inhibitors can be used for the treatment of many diseases. Carbonic anhydrase inhibitors are the first line treatments for glaucoma, cancer, osteoporosis, obesity, neurological disorders, as well as for gastric and duodenal ulcers. It may also act as diuretics and antiepileptic (8).

Many studies have been conducted to find the effects of N. jatamansi in CNS diseases (9). A literature search found that carbonic anhydrase inhibition and antibacterial activity of Nardostachys jatamansi methanolic extract against selected bacterial strains have not been reported so far.

In this study, antibacterial activity against some selected bacterial strains and carbonic anhydrase inhibition of N. jatamansi methanolic extract are described for the first time providing the basis for research to find new entities against different diseases.

EXPERIMENTAL

Reagents
Tris (Invitrogen: cat# 15504-020), HEPES (bioworld: cat#40820000-1), carbonic anhydrase (Sigma-Aldrich, C2624, P-Code: 1001584424), 4-nitrophynol acetate (Sigma-Aldrich, N8130, lot#BCBK4587V), acetazolamide (Sigma-Aldrich, Lot BCBK5191V, P-Code 101400375, ≥ 99% powder), dimethyl sulfoxide (Merck, Germany), nutrient agar (Merck, Germany), nutrient broth (Merck, Germany), Ciproflaxacin (Novidat), Registration # 012066, Sami Pharmaceuticals (Pvt.) Ltd.

Apparatus
Micro plate reader (Synergy HT BioTek® USA), Digital rotary evaporator apparatus, (Heidolph Laboratory, Germany), pH meter (WTW series Inolab), digital weighing balance (Uni Bloc, Shimadzu, AUW220D).

Collection and identification of plant sample
Dried plant material was purchased from the local market and identified by the botanist, Dr. Sarwar, Lecturer, The Islamia University, Bahawalpur (Voucher No. 2205/L.S). The voucher specimen was deposited in Botany Department, The Islamia University, Bahawalpur, Pakistan.

Preparation of extract
The plant extract was prepared by maceration method. Plant material was powdered in the electric grinder; 100 g of dried powdered plant material was taken in the amber colored glass bottle and 400 mL of methanol was added to it. The material was soaked for 15 days with occasional shaking. After 15 days, the soaked material was filtered through muslin cloth and then by Whatman # 1 filter paper by using Buchner funnel. The process was repeated three times with 200 mL methanol to extract maximum contents and material (10). The solvent was evaporated by using rotary evaporator. The remainder was collected in little glass bottles and stored at 4℃ until next use. It was the crude methnolic extract of N. jatamansi rhizomes.

A stock solution of freeze dried extract 5 mg plant extract/mL of DMSO was prepared and serial dilutions in the range of 0.5-5 mg/mL prepared from the stock solution.

Preliminary phytochemical screening
N. jatamansi rhizome extract was subjected to preliminary qualitative phytochemical screening for alkaloids (Dragendorff’s and Mayer’s test), flavonoids (sodium hydroxide), tannins (ferric chloride test) and phenols (ferric chloride test) (11).

Bacterial strains and growth media
Staphylococcus aureus (S.A) ATCC-6538, Pseudomonas aeruginosa (P.A) ATCC-9027, purchased from Microbiologics Inc. Escherichia coli (E.C), Klebsiella pneumonia (K.P), Shigella dysenteriae (S.D), Corynebacterium striatum (C.S), Bacillus subtilus (B.S), Proteus vulgaris (P.V) purchased from first fungal culture Bank of Pakistan (FCBP), Institute Of Agricultural Sciences, University Of The Punjab, Lahore, Pakistan; accession numbers were 12, 14, 72, 147, 174, 368, respectively. All the bacterial strains were grown on nutrient agar at 37℃ for 24 h.
Synthetic antibacterial and carbonic anhydrase inhibitor

Ciprofloxacin (Novidat 200/100 mg/mL) was used as standard antibacterial drug and acetazolamide was used as a standard carbonic anhydrase inhibitor.

Preparation of inoculum

Nutrient agar media were prepared by dissolving 28 g of nutrient agar powder in 1000 mL of distilled water, heated until bubbles appeared and then sterilized at 121°C and 15 psi in autoclave for 15 min. Bacterial inoculums were prepared from 24 h old pure culture on nutrient agar. Bacterial colonies were grown in nutrient broth for 24 h. In 1 liter of distilled water, 8 g of broth was dissolved and kept in an autoclave (for sterilization) for 20 min at 121°C at 15 psi. In Erlenmeyer flasks, 50 mL of broth and 50 µL of culture stock solution was added and mounted on the horizontal shaker. After 24 h, cell turbidity was checked spectrophotometrically in comparison to that of 0.5 McFarland standard. Then, the inoculum was used for the antibacterial activity.

In vitro susceptibility tests

Agar well diffusion method

The agar well diffusion method was performed for the determination of antibacterial activity of N. jatamansi methanolic extract. Prior to being tested for the antibacterial activity, crude plant extract was dissolved in DMSO and stock solution of 5 mg/mL was prepared. The experiment was performed by the method of (12) with slight modifications. 20 mL of Mueller Hinton agar was placed in Petri dishes and allowed to solidify. A suspension of the microorganism of 60 µL was evenly spread on the surface of Mueller Hinton agar with sterile cotton-tipped swab. Wells of 6 mm in diameter were made on solid agar surface with the help of cork borer in each Petri dish. 20 µL of extract solution was added to each well. Petri dishes were placed in the incubator at 37°C for 24 h. After 24 h, the zones of inhibitions were measured to estimate the antibacterial activity. The experiment was done in triplicate and results were taken as an average of the three tests. The test was also performed with standard ciprofloxacin and methanol.

Broth micro dilution method

Antibacterial analysis and determination of minimum inhibitory concentration (MIC) of the extract and ciprofloxacin against different bacterial strains were performed by broth micro dilution method. This assay was carried out by the method of (13) with slight modifications. The test was performed in sterile 96-well micro plates. Total mixture volume in a well was 200 µL, contained 20 µL of methanolic extract solution and 180 µL suspension of bacterial culture. At 540 nm absorbance was measured and this was taken as pre read. Then for 16-24 hours, the plates were incubated at 37°C. After read was measured at 540 nm and the difference between pre read and after read was taken as an index of bacterial growth. All readings were taken as triplicate. Results are mean of triplicate (n = 3, ± S.E.M). Standard drug was ciprofloxacin and instead of test sample methanol was added in the assay as the negative control.

The % inhibition was calculated by the following formula

\[
\text{Inhibition} (\%) = 100 \times \left( X - Y \right) / X
\]

where

\[
X = \text{absorbance in negative control with bacterial culture}
\]

\[
Y = \text{absorbance in test sample with bacteria}
\]

Serial dilutions of the test samples were made to calculate the minimum inhibitory concentration. EZ-Fit5 Perrella Scientific Inc. Amherst USA software was used for MIC calculation.

Carbonic anhydrase inhibition assay

Carbonic anhydrase inhibition assay was performed according to (8) with slight modification. In this test, the formation of 4-nitrophenol was measured, that is a yellow color compound. 4-Nitrophenol was formed by the hydrolysis of 4-nitrophenyl acetate. The experiment was performed in 20 mM buffer of 7.4 pH containing Tris and HEPES. For each sample reaction well included following items: 140 µL of buffer, 20 µL of the freshly prepared solution of enzyme (0.1 mg/mL of deionized water) of purified bovine erythrocyte CA-II and 20 µL of the test compound. The test compound was incubated for 15 min at 25°C and pre-read was taken at 400 nm by using Synergy HT BioTek® USA micro plate reader.

The reaction started by the addition of 4-nitrophenyl acetate. 4-Nitrophenol acetate was added in 20 µL at the concentration of 0.7 mM, diluted in ethanol and incubated at the same conditions for 30 min and after read was taken at 400 nm. The reaction performed in triplicate with different concentrations. Assay was also performed with methanol for possible inhibition of enzyme as the extraction of plant was done with methanol. Percent inhibition was measured by formula given below;

\[
\% \text{ inhibition} = 100 - \left( \text{absorbance of test compound/absorbance of control} \right) \times 100
\]
Statistical analysis
All the measures were done in triplicate and results were expressed as the mean ± S.E.M. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis. A p-value ≤ 0.05 was considered significant.

RESULTS
Preliminary phytochemical analysis of extract showed the presence of alkaloids, flavonoids, tannins and phenols.

The growth inhibition value of methanolic extracts of N. jatamansi on different bacterial strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Zone of inhibition (mm) mean ± SEM*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>12.6 ± 0.5</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>10.3 ± 0.5</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Corynebacterium striatum</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>11 ± 0.2</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>8 ± 0.5</td>
</tr>
</tbody>
</table>

*SEM, standard error of mean: a Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 32 ± 1; b Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 35 ± 0.5; c Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 35 ± 1; d Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 30 ± 1; e Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 26 ± 1; f Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 30 ± 1; g Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 32 ± 0.5.

Table 2. MIC of methanolic extract of N. jatamansi and ciprofloxacin against bacterial strains.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>N. jatamansi MIC (mg/mL)</th>
<th>Ciprofloxacin MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.5</td>
<td>0.015</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td>Corynebacterium striatum</td>
<td>0.6</td>
<td>8</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.6</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3. In vitro carbonic anhydrase inhibitory activity of N. jatamansi methanolic extract.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>IC50 (Mean ± S.E.M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. jatamansi</td>
<td>712.41 ± 0.001 µg/mL</td>
</tr>
<tr>
<td>Acetazolamide (Standard)</td>
<td>0.03 ± 0.00 µg/mL or 0.14 µM</td>
</tr>
</tbody>
</table>

*IC50 readings are mean ± S.E.M of 3 readings
is shown in Table 1. All bacterial strains showed marked antibacterial activity. The diameters of growth of inhibition zone of extract at various concentrations were between 8-22 mm. The diameters of the zone of inhibitions were decreased by reducing the concentration of extract. Ciprofloxacin showed zone of inhibitions in a range of 26-35 mm. The methanolic extract showed the highest inhibition against *Bacillus subtilis* and least against *Shigella dysenteriae*. However, methanol showed no inhibition zone against any bacterial strain. There was a significant difference between the zone of inhibition values of ciprofloxacin and extract (p < 0.05).

Table 2 summarizes the results of antibacterial activity as MIC through broth microdilution method. The MIC values of extract ranged from 0.3-0.7 mg/mL. Results showed that the highest inhibitory activity of the plant extract was found against *Bacillus subtilis* (MIC 0.3 mg/mL) and the lowest against *Shigella dysenteriae* (MIC 0.7 mg/mL). The results obtained from ciprofloxacin showed resistance to all selected bacterial strains representing MIC ranged from 0.015-8 µg/mL. There was a significant difference between MIC values of ciprofloxacin and extract (p < 0.05). It is evident from the results of the study that the highest the zone of inhibition, the lowest the minimum inhibitory concentration. In present study, the *N. jatamansi* extract showed highest zone of inhibition (22 mm) with the lowest MIC (0.3 mg/mL) against *Bacillus subtilis*. The lowest zone of inhibition was against *Shigella dysenteriae* (17 mm) with MIC (0.7 mg/mL).

In this study, *N. jatamansi* methanolic extract showed a marked inhibition of carbonic anhydrase (IC$_{50}$ 712.41 µg/mL) as represented in Table 3. Maximum inhibition of carbonic anhydrase (87%) was with the stock solution of the methanolic extract (1 mg/mL in DMSO). Methanol has no effect on carbonic anhydrase inhibition.

**DISCUSSION**

Drugs from natural sources are preferred because they perceived drug likeness and biological friendliness than synthetic compounds (14). Drug resistance due to an inappropriate and wide use of synthetic medicines is a major issue. Moreover, there are various side effects of these commercially available drugs (15). The study was conducted to investigate antibacterial activity of medicinal extract of *N. jatamansi* against *S. aureus, P. aeruginosa, E. coli, K. pneumoniae, S. dysenteriae, C. striatum, B. subtilis, P. vulgaris*, common clinical bacteria that can cause infection in humans. Antibacterial activity of different types of extract of *N. jatamansi* was reported against some bacterial strains (16), the screening of methanolic extract on all these bacterial strains and their minimum inhibitory concentrations (MICs) were not revealed before.

Based on agar well diffusion assay results of *B. subtilis, S. aureus, K. pneumoniae* showed the highest antibacterial activity while others showed moderate antibacterial activity. The similar findings were reported by (16) that the mixture of dichloromethane and methanol extract of *N. jatamansi* showed marked inhibition of *B. subtilis, S. aureus, K. pneumoniae*. However, Kumar (16) reported the lack of activity of essential oil and mixture of dichloromethane and methanol extract of *N. jatamansi* against *P. aeruginosa* and *E. coli*.

It has been frequently reported that antibacterial activity of medicinal plants is mostly due to the presence of alkaloids, flavonoids, tannins and triterpenoid in the plant extracts (17). The phytochemical evaluation of this extract has proved the existence of alkaloids, flavonoids and tannins in the methanolic extract, which might be the possible reason lying behind antimicrobial potential of this extract. The presence of alkaloids and tannins in methanolic extract of *N. jatamansi* was also reported in another work (4).

Antibacterial activity of extract against different bacterial strains was dose dependent. Activity was decreased by decreasing the concentration of extract. *N. jatamansi* extract has antibacterial effects may be by direct action of the extract on structure and metabolism of bacteria. The study was the first one that determined MIC of *N. jatamansi* extract. The role of MIC determination lies in the fact that lower the MIC, more chances of it being useful for medicinal purposes. The lower the doses required to achieve therapeutic effect have chances of lower toxicity and side effects (18).

*Staphylococcus aureus* skin and soft tissue infections cause minor boils or abscesses and can lead to severe infections as endocarditis (19). The roots of *N. jatamansi* are used traditionally in the treatment of boils and itch, etc. (2). Results of this study showed that *N. jatamansi* extract has marked inhibition against *S. aureus* (MIC 0.4 mg/mL) thus providing the basis for its traditional use in boils through antibacterial effects.

The results of present study presented the marked inhibition of carbonic anhydrase, so it can be speculated that traditional effects of *N. jatamansi* as diuretic, antiepileptic effect and in eye diseases may be due to carbonic anhydrase inhibition. *N.
jatamansi extract can be used for clinical treatment of various diseases as a syrup. Formulation containing N. jatamansi as an important ingredient has reduced febrile convulsions in children during an experimental clinical trial (20). So, traditional antiepileptic effect of N. jatamansi that is proved in current study through in vitro carbonic anhydrase potential was also confirmed by a clinical trial. Moreover, anticancer effects of N. jatamansi that can be speculated from the current study due to carbonic anhydrase inhibitory potential is supported by cytotoxic activity of N. jatamansi extract against lung and prostate cancer cell lines (21).

CONCLUSION

N. jatamansi inhibited carbonic anhydrase sufficiently and showed significant antibacterial activity against different bacterial strains. Thus, the traditional use of it in various diseases is related to its reported activities. Moreover, the results of the study suggest that N. jatamansi may be a valuable plant source of medicinally active constituents that can be useful for the treatment of diseases.

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Liver, the second largest organ of body responsible for a number of important functions including production of bile, urea and plasma proteins, excretion of bilirubin, cholesterol, hormone and drugs and also metabolism of protein, vitamin, minerals, carbohydrates and fats (1), is most susceptible to toxicity (2). Hepatotoxicity is caused by chemicals that damage hepatic vasculature, hepatocytes and biliary epithelial cells either directly or indirectly by producing their reactive metabolic species, hence eliciting an immune reaction and resulting in hepatic injury (3). Most of the toxins have minimum liver damaging potential which can be reversed by terminating the use of offending agent. In overdose, causative mediator results in hepatic necrosis and if not treated timely and effectively, may lead to death (3). Moreover, it is estimated that over 900 drugs have potential to cause hepatic damage that is why most marketed drugs with hepatotoxic potential have been withdrawn (4). Drug induced liver injury can be either intrinsic or idiosyncratic (4) and results from inhalation, ingestion and parenteral administration of drugs. Certain drugs cause hepatic injury even at therapeutic doses (5), for instance paracetamol, diclofenac, isoniazid, halothane, erythromycin, penicillamine and phenytoin. Moreover, reactive metabolites of various drugs interact with essential macromolecules such as proteins, lipids or nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage and oxidative stress and finally resulting in hepatic damage (6). Around the world, every year about 20,000 deaths occur followed by hepatic diseases (5).

Current therapy of liver disorders include interferons, lamivudine, adefovir, ribavirin and corticosteroids. But these medications owe many limitations such as low efficacy, high risk of adverse effects and high price. Therefore, use of herbal remedies in treating liver maladies has been increasing nowadays. Various herbs are known for pos-

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**EVALUATION OF HEPATOPROTECTIVE POTENTIAL OF EUPHORBIA PROSTRATA AIT EXTRACT AGAINST CHEMICALLY INDUCED HEPATOTOXICITY IN ALBINO MICE**

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2Drug Control Office Gilgit-Baltistan, Pakistan
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**Abstract:** Hepatic diseases are serious health issues as various pharmaceuticals, xenobiotics, environmental/chemical pollutants, some natural products and dietary supplements when used in overdose have tendency to cause hepatotoxicity. Present study was aimed at investigating hepatoprotective activity of aqueous methanolic extract of *Euphorbia prostrata* against paracetamol and carbon tetrachloride induced hepatotoxicity in mice, using silymarin as a standard drug. Hepatoprotective effects were accessed by investigating serum marker enzymes (aspartate transaminase, alanine transaminase, alkaline phosphatase), total bilirubin, albumin and total protein as well as histopathological analysis. *Euphorbia prostrata* exhibited significant reduction in serum marker enzymes at 250 mg/kg and 500 mg/kg doses. Moreover, histopathological studies also supported biochemical estimation. It could be concluded that aqueous methanolic extract of *Euphorbia prostrata* possesses hepatoprotective potential against carbon tetrachloride and paracetamol induced hepatic injury which might to due to anti-oxidant activity displayed by flavonoids and polyphenolics.

**Keywords:** *Euphorbia prostrata*, hepatotoxicity, marker enzyme, paracetamol, carbon tetrachloride

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sessing hepatoprotective properties, derived from different cultural sources (7). *Euphorbia prostrata* Ait is an annual herb, mainly found in tropic and subtropic areas naturally present in Asia, Africa and many other parts of world. It is used traditionally as anti-oxidant (8) and to inhibit HIV-1 and hepatitis C virus proteases (9, 10). It has been validated for pharmacological effects like anti-inflammatory activity (11), hemorrhoid (12), diarrhea (13), antibacterial, anti-fungal activity (14) and diabetes (15). The purpose of present study was to pharmacologically investigate the hepatoprotective effect of *Euphorbia prostrata* using animal models of liver injury thus supporting its customary use in liver ailments.

**MATERIALS AND METHODS**

**Chemicals**

The chemicals used were methanol, chloroform, ether, normal saline, paracetamol, carbon tetrachloride, silymarin (all from Sigma Aldrich). All the other chemicals used were of analytical grade.

**Plant material**

The aerial parts of *Euphorbia prostrata* Ait (2 kg) were collected from Dhillam Ballaggan, Sialkot, Punjab, Pakistan from April to May 2015. Plant was identified and authenticated by Professor Dr. Ashiq, Department of Botany, University of Agriculture, Faisalabad. The washed and shade dried plant material was ground into powder with a Chinese herbal grinder.

**Preparation of plant extract**

Aqueous methanolic (30 : 70) extract of aerial parts of *Euphorbia prostrata* was prepared using cold maceration technique. Coarse powder of plant was soaked in 3 L of 70% methanol and kept at room temperature for 3 days (72 h) with occasional stirring daily, followed by filtration after 3 days. This process was repeated thrice. Afterward, all the filtrates were combined and again filtered through muslin cloth and Whatman filter paper I. The filtrate was dried and concentrated under reduced pressure in rotary evaporator at 50°C. The solid extract thus formed was stored in a capped container in refrigerator. The color of crude extract was dark brown (16).

**Animals used**

Young and healthy albino mice (20-40 g) of both sexes (4-5 months) were used. The mice were housed under standard conditions of temperature (23 to 25°C), relative humidity (55%) with 12 h light and 12 h dark cycle at animal house of University of Sargodha, Sargodha. They were fed with standard pellet diet and tap water *ad libitum*. All the experiments performed complied with the rules of National Research Council (17).

**Paracetamol (PCM) induced hepatotoxicity**

In the dose response experiment, albino mice were randomly divided into 5 groups (n = 4). Group I (Normal control group) animals received normal saline 1 mL/kg, p.o. for 7 days. Group II (Diseased control) mice were given normal saline, 1 mL/kg p.o. for 7 days. Group III (Standard group) mice were administered silymarin, 100 mg/kg p.o. for 7 days. Group IV and V (Experimental groups) animals were given *Euphorbia prostrata* extract, 250 mg/kg/d and 500 mg/kg/d p.o. for 7 days, respectively.

On 7th day, 1 h after administering normal saline (disease control group), silymarin, 250 mg and 500 mg of *Euphorbia prostrata* extract to Group II, III, IV and V, respectively, paracetamol (250 mg/kg) was given orally. After 24 h of administration, mice were sacrificed under mild ether anesthesia and hepatoprotective activity was assessed (18).

**Carbon tetrachloride (CCL4) induced hepatotoxicity**

Albino mice were randomly divided into 5 groups (n = 4). Group I (Normal control group) was given distilled water 1 mL/kg p.o. for 7 days followed by administration of olive oil (1 mL/kg, s.c.) on 7th day, 1 h after feeding distilled water. Group II (Diseased control) mice were administered distilled water (1 mL/kg, p.o.) for 7 days. Group III (Standard group) animals received silymarin, 100 mg/kg p.o. for 7 days. Group IV and V (Experimental groups) mice were given *Euphorbia prostrata* extract, 250 mg/kg/d and 500 mg/kg/d p.o. for 7 days, respectively.

On 7th day, 1 h after administration of distilled water, sylimarin, 250 mg and 500 mg of *Euphorbia prostrata* extract to Group II, III, IV and V, respectively, carbon tetrachloride (20% v/v in olive oil) 1 mL/kg was given subcutaneously. After 24 h of administration, mice were sacrificed under mild ether anesthesia and hepatoprotective activities were evaluated (19).

**Assessment of hepatoprotective activity**

The hepatoprotective activity was appraised biochemically as well as histopathologically. After
4 h of drug treatment, animals were anesthetized using ether. Blood was withdrawn from each mouse through carotid artery and collected in centrifugation tubes and was allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 3000 rpm for 15 min and biochemical parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, bilirubin and total protein were evaluated. The hepatic tissues were also excised quickly, washed with saline and stored in 10% formalin. Four representative sections were taken and submitted in one block. The histopathological examination of these sections revealed architecture of liver (5).

Statistical analysis

The results were expressed as the means ± standard error of mean (S.E.M). One-way ANOVA followed by Dunnet test was applied using Graphpad prism software. Significance was set at 95% (p < 0.05).

RESULTS

The present study revealed that in paracetamol induced intoxication, Euphorbia prostrata extract exhibited highly significant reduction in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (p < 0.01) and alkaline phosphatase (ALP) (p < 0.001) at 250 mg/kg dose. Moreover, Euphorbia prostrata extract caused highly significant (p < 0.001) reduction in activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) at 500 mg/kg as compared to disease control group. However, Euphorbia prostrata extract produced non-significant changes in levels of alkaline phosphatase (ALP) at 500 mg/kg. Also, non-

Table 1. Effect of methanolic extract of Euphorbia prostrata on biochemical parameters in paracetamol induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total bilirubin (mg/dL)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
<th>Albumin (g/dL)</th>
<th>Total protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCM (250 mg/kg)</td>
<td>0.83 ± 0.01</td>
<td>82.66 ± 15.45</td>
<td>85.33 ± 18.98</td>
<td>209.33 ± 5.20</td>
<td>4.30 ± 0.88</td>
<td>7.53 ± 0.62</td>
</tr>
<tr>
<td>Normal control</td>
<td>0.53 ± 0.06</td>
<td>79.33 ± 4.05</td>
<td>72.33 ± 2.33</td>
<td>135.66 ± 5.36</td>
<td>3.00 ± 0.05</td>
<td>5.86 ± 0.46</td>
</tr>
<tr>
<td>Silymarin + PCM</td>
<td>0.53 ± 0.03</td>
<td>19.00 ± 1.52</td>
<td>14.66 ± 1.85</td>
<td>193.33 ± 4.41</td>
<td>3.90 ± 0.57</td>
<td>6.53 ± 0.31</td>
</tr>
<tr>
<td>EP + PCM (250 mg/kg)</td>
<td>0.46 ± 0.03</td>
<td>50.00 ± 4.58</td>
<td>40.66 ± 2.33</td>
<td>234.66 ± 6.38</td>
<td>3.93 ± 0.58</td>
<td>7.60 ± 0.51</td>
</tr>
<tr>
<td>EP + PCM (500 mg/kg)</td>
<td>0.60 ± 0.00</td>
<td>21.00 ± 0.57</td>
<td>32.00 ± 1.73</td>
<td>206.00 ± 12.74</td>
<td>2.83 ± 0.06</td>
<td>6.13 ± 0.13</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM (n = 4), where, ns = (p > 0.05), * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) vs. PCM (250 mg/kg). PCM=Paracetamol, EP=Euphorbia prostrata

Table 2. Effect of methanolic extract of Euphorbia prostrata on biochemical parameters in carbon tetrachloride induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total bilirubin (mg/dL)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
<th>Albumin (g/dL)</th>
<th>Total protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCl4 (1 mL/kg)</td>
<td>1.60 ± 0.15</td>
<td>175.00 ± 15.37</td>
<td>72.00 ± 16.16</td>
<td>441.00 ± 28.93</td>
<td>6.70 ± 1.35</td>
<td>9.33 ± 0.17</td>
</tr>
<tr>
<td>Normal control</td>
<td>0.53 ± 0.06ns</td>
<td>79.33 ± 4.05</td>
<td>72.33 ± 2.33</td>
<td>135.66 ± 5.36</td>
<td>3.00 ± 0.05</td>
<td>5.86 ± 0.46</td>
</tr>
<tr>
<td>Silymarin + CCl4 (100 mg/kg/10mL)</td>
<td>0.63 ± 0.03</td>
<td>47.66 ± 4.63</td>
<td>42.33 ± 9.06</td>
<td>162.00 ± 7.57</td>
<td>3.10 ± 0.10</td>
<td>5.83 ± 0.44</td>
</tr>
<tr>
<td>EP + CCl4 (250 mg/kg)</td>
<td>0.50 ± 0.05</td>
<td>51.66 ± 5.36</td>
<td>42.66 ± 1.76</td>
<td>186.66 ± 4.41</td>
<td>3.46 ± 0.27</td>
<td>6.46 ± 0.12</td>
</tr>
<tr>
<td>EP + CCl4 (500 mg/kg)</td>
<td>0.63 ± 0.03</td>
<td>34.66 ± 2.40</td>
<td>29.00 ± 1.73</td>
<td>158.66 ± 4.66</td>
<td>3.20 ± 0.15</td>
<td>6.30 ± 0.25</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SEM (n = 4), where, ns = (p > 0.05), * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) vs. CCl4 (1mL/kg), CCl4=carbon tetrachloride, EP=Euphorbia prostrata
significant changes in levels of total bilirubin, albumin and total protein at both concentrations were observed as illustrated in Table 1.

Likewise, in carbon tetrachloride induced hepatotoxicity model, methanolic extract significantly (p < 0.001) decreased the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) at both doses but there were non-significant changes in the levels of bilirubin, albumin and total protein at both doses as presented in Table 2.

Additionally, histopathologic studies demonstrated that, in normal control group, normal architecture of hepatocytes was observed with intact cell nuclei and regular portal vein. While, in paracetamol intoxicated group, severe histopathological changes were observed such as infiltration of inflammatory cells, fatty changes, necrosis and ballooning degeneration of hepatocytes, granuloma and malignancy. Silymarin (standard) treated group unveiled that hepatocytes were arranged in single plate with central veins and porta hepatis and no inflammatory or necrotic changes were noticed in standard group. Moreover, no granuloma or malignancy was evident. While, among extract treated groups, 250 mg/kg exhibited partial protection of hepatocytes and prevented histopathological changes associated with hepatotoxicity induced by acetaminophen, but hepatocytes were mildly infiltrated by inflammatory cells. However, no necrosis, granuloma or malignancy was seen. While, at 500 mg/kg, hepatocytes were arranged in single plate, central vein and porta hepatis were also clearly discernible. There were no signs of inflammatory and necrotic changes, granuloma or malignancy (Fig. 1).

The liver sections of carbon tetrachloride treated mice divulged characteristic centrilobular pattern of degeneration, liver fibrosis, hyperemia around central vein, wide vacuolar degeneration of hepatocytes, lymphocyte infiltration, derangement of hepatocyte cord and necrosis at periphery of central vein. Whereas, in normal control group, central vein, portal space and hepatocytes were normal. However, silymarin distinctly protected liver from carbon tetrachloride induced damage as revealed by histopathological analysis. Similarly, *Euphorbia prostrata* extract provided hepatic protection comparable to standard group particularly at 500 mg/kg dose (Fig. 2).

**DISCUSSION**

Liver is the central organ having astounding role in metabolism, excretion and detoxification of...
chemotherapeutic agents, xenobiotics and environmental pollutants. It is involved in maintenance, regulation of body homeostasis and various biochemical pathways to growth, nutrient provision and fight against disease, energy generation and reproduction. Various liver maladies like hepatitis, cirrhosis and fatty liver disease originate because of exposure to environmental toxins, generation of free radicals, deprived food habits, alcohol abuse and numerous prescribed and over-the-counter drugs (20). The available synthetic drugs are less effective in treating hepatic diseases owing to their numerous adverse effects and low therapeutic potential. Therefore, use of herbal medicines has been increasing gradually in both developed as well as developing countries as they are economical with substantial biological effects and free from toxic profile. Furthermore, plant based preparations play a noteworthy role in regeneration of liver cells and acceleration of healing process and hence management of numerous liver ailments (21). Currently, most effective therapy for hepatic ailments are constituents derived from herbal drugs such as glycyrrhizin (Glycyrrhiza glabra) and silymarin (Silybum marianum) in Japan, catechin (Anacardium occidentalis) in Europe, and chizandrins (Schizandra chinensis) in China (22). Similarly, in present study, hepatoprotective activity of aqueous methanolic extract of Euphorbia prostrata was evaluated using paracetamol (PCM) and carbon tetrachloride (CCL4) as hepatotoxins to scientifically support its folkloric use.

These hepatotoxic agents cause liver damage resulting in elevation of marker enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Carbon tetrachloride is a hepatotoxic agent that undergoes biotransformation in endoplasmic reticulum by CYP 450 and produces trichloromethyl free radical i.e. CCl3 that interact with cellular proteins and lipids. The trichloromethylperoxyl radical more potentially attacks lipids of endoplasmic reticulum causing lipid peroxidation and alter calcium homeostasis resulting in cell death and release of enzymes in circulation. In current study, methanolic extract of Euphorbia prostrata markedly decreased levels of liver marker such as enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), total bilirubin and albumin comparable to that of standard drug silymarin. However, in carbon tetrachloride intoxication, Euphorbia prostrata extract reduced the levels of total bilirubin in dose independent manner, which might be due to either enzyme/receptor saturation or genetic variations. Likewise, paracetamol is a well-known and most commonly used analgesic and anti-pyretic drug. Hepatotoxic doses of paracetamol deplete normal stores of hepatic glutathione. CYP 450 enzymes such as CYP2E1 and CYP1A2 metabolize paracetamol and forms NAPQI (N-
acetyl-p-benzo-quinoneimine) that is an alkylating metabolite. The P450 gene is highly polymorphic in nature as CYP2D6, third isoenzyme, is associated with individual variation towards paracetamol toxicity. Paracetamol is metabolized into NAPQI to a lesser extent by CYP2D6 but in ultra-rapid metabolizers, this enzyme causes hepatotoxicity. Glutathione is a natural anti-oxidant, but in paracetamol toxicity NAPQI becomes irreversibly conjugated with sulfhydryl group of glutathione, thus causing its depletion. NAPQI causes hepatic damage by releasing inflammatory mediators such as TNF-α, responsible for tissue necrosis (23). In paracetamol intoxicated mice, plant extract showed a dose dependent decrease in the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), which might be due to stabilization of plasma membrane, as well as repair of hepatic tissue damage caused by carbon tetrachloride and paracetamol (24). Moreover, plant extract significantly decreased albumin level, but surprisingly it exhibited concentration independent behavior in case of total bilirubin which might be due to hereditary disparity or receptor saturation producing less than expected response at higher dose as in carbon tetrachloride model. However, in previous studies it has been revealed increase in serum total bilirubin level to be owing to defective bile excretion by liver, associated with loss of integrity of liver and eventually tissue necrosis. Hence, this results in increase in binding, conjugating and excretory capacity of liver cells, relative to erythrocyte degeneration rate. Moreover, depletion in serum bilirubin level by plant extract might be due to its ability to alleviate biliary dysfunction during paracetamol induced hepatotoxicity in prophylactic studies (24). Besides, histopathological examination also supported hepatoprotective ability of Euphorbia prostrata.

Since, it has been found that flavonoids through their free radical scavenging activity are accountable for hepatoprotection. Thus, liver damage induced by carbon tetrachloride and paracetamol is oxidative in nature, usually reversed by anti-oxidants by stabilizing cell membrane and repairing liver tissue damage. Moreover, various studies revealed hepatoprotective action of alkaloids due to their anti-oxidant property (25). Also, hepatoprotective agents act by inhibiting aromatase activity of CYP 450 which favors liver regeneration (26). Hence, current study has revealed hepatoprotective aptitude of Euphorbia prostrata against carbon tetrachloride (CCL4) and paracetamol (PCM) induced liver damage, which could be due to its anti-oxidant activity, as it contains glycosides, flavonoids, polysaccharides, anthraquinones, phlobatannins, saponins and various other alkaloids (27, 28). Furthermore, in another study it has been ascertained that aqueous methanolic extract of E. prostrata possess noticeable scavenging properties and scavenge DPPH, which might be owing to the presence of phenolic and polyphenolic compounds that significantly inhibit oxidative stress caused by free radicals (29). Moreover, it has been avowed that Euphorbia thymifolia possesses hepatoprotective potential due to marked anti-oxidant and inhibitory lipid peroxidation (LPO) activity (30, 31). So, Euphorbia prostrata being the member of same family and genus might have exerted hepatoprotective effect due to its strong anti-oxidant and lipid peroxidation inhibition activity and owing to the presence of flavonoids and phenolics. Further studies and investigations are required to prove hepatoprotective potential of active constituents of plant extract.

CONCLUSION

Euphorbia prostrata is a medicinally valuable plant and its hepatoprotective activity might be due to flavonoid and phenolic constituents which exhibit anti-oxidant and anti-lipid peroxidation properties. However, actual mechanism is not known and it needs to be investigated.

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The authors are thankful to University of Sargodha for providing all chemicals and material required for research work and also grateful to staff of Animal House of University Sargodha for their help and support.

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Lovastatin (C\textsubscript{24}H\textsubscript{36}O\textsubscript{5}) is a potent drug that is used to lower blood cholesterol level of humans. It actually belongs to a group of fungal secondary metabolites known as statins which also include various other cholesterol-lowering drugs such as pravastatin, simvastatin, mevastatin, etc. Lovastatin and pravastatin are natural statins; simvastatin is semi-synthetic while atorvastatin and fluvastatin are synthetic statins. Natural statins can be produced through microbial fermentation (1). Lovastatin is a reversible competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase which catalyzes a rate-limiting step in the biosynthesis of cholesterol (Fig. 1A & B) (2). They are the most efficient agents for reducing plasma cholesterol, being also appreciated for their good tolerance. The beneficial effects of the HMG CoA reductase inhibitors are usually attributed to their capacity to reduce the endogenous cholesterol synthesis (3). Since mevalonate, the product of HMG CoA reductase reaction is the precursor not only for cholesterol, but also for many other nonsteroidal isoprenoidic compounds, inhibition of this key enzyme may result in pleiotropic effects (4).

Lovastatin was isolated from Penicillium citrinum and Penicillium brevicespactum (5). Among the genus Aspergillus, the most important species for statin production are A. terreus, A. flavipes, A. flavus, A. umbrosus, and A. parasiticus (4, 6). However, some hyper-producing strains of A. terreus produce high amount of lovastatin under submerged fermentation, but it is not generally considered as safe drug and not suitable for direct consumption by human beings. Liquid state fermentation is not preferably chosen due to low yield, extensive downstream processing and the consequent high capital and high operating expenses therefore, now it is replaced by solid state fermentation (6). The UV radiation is the most convenient of all mutagens to use, and it is also very easy to take effective safety precautions against it. It gives a high proposition of pyrimidine dimmers and includes all types of base pair substitutions (7). Cell immobilization is considered a promising approach for enhancing the fermentation processes. It facilitates continuous operation over a prolonged period, offers possible recycling of immobilized beads and simple way for harvesting of products, reactor pro-

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**STRAIN IMPROVEMENT OF ASPERGILLUS TERREUS FOR HYPER-PRODUCTION OF LOVASTATIN AND IMMOBILIZATION OF MUTANT ATU-06 STRAIN FOR REPEATED BATCH CULTURE PROCESS**

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Abstract: The aim of this work was to hyper-produce lovastatin by strain improvement of *Aspergillus terreus* S-24 through UV mutagenesis using corn cobs as carrier substrate in solid state fermentation. Ten positive mutants were selected on agar plates for still plate experiments. ATU-06 mutant strain showed 45.63 ± 1.74 mg/100 mL lovastatin concentration higher than parent strain (18.74 ± 1.68 mg/100 mL) in pre-optimized fermentation medium. Fermentation parameters pH (6), temperature (30\(^\circ\)C), inoculum size (2 mL) moisture contents (60\%) and incubation time (120 h) were optimized for both mutant and parent strains. Kinetic growth parameters also showed that mutant strain ATU-06 showed greater stability and improved results as compared to parent strain. Finally, the mutant *Aspergillus terreus* ATU-06 strain was immobilized by entrapping it in calcium alginate beads and agar for repeated use.

Keywords: mutation, lovastatin, *Aspergillus terreus*, immobilization, kinetic parameters

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ductivity and higher efficiency of catalysis and development of economical methods for lowering operational cost of industrial processes (8).

Therefore, the present study was conducted for hyper production of cholesterol lowering drug lovastatin produced by improving *Aspergillus terreus* strain on exposure to UV irradiation. Strain improvement for the production of alkaline lovastatin has been reported by few scientists but there is an information gap regarding use of these inexpensive materials for considerably high production of value added products by strain improvement and the immobilization of mutant cells for repeated batch culture or multiple uses of these immobilized cells.

EXPERIMENTAL

Microorganism procurement and culture maintenance

The pure local culture strain of *A. terreus* S-24 was obtained from Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. The culture of *A. terreus* S-24 (stock culture) was maintained in a refrigerator (4°C) on nutrient agar medium (Oxoid, Hampshire, UK).

Mutagenesis by UV lamp

For hyper-production of lovastatin, the spores of *A. terreus* S-24 (1 × 10⁷ spores/mL) were mutated by using UV lamp (UV-A, 20W, Phillips, λ₃60, distance of the lamp from the Petri plates was 20 cm). These spores (10 mL) were exposed to ultraviolet light. The sample (1 mL) was withdrawn after 30 min duration till 180 min. The optimum dose was selected by preparing the survival data (9).

Selection of mutant

In the dark room, the 0.1 mL of these spore dilutions were streaked on nutrient agar plates (Oxoid, Hampshire, UK), that contain 0.1% triton X-100 to inhibit the growth of other fungal colonies. All these plates were kept at 37°C for 48 hours in an

![Figure 1. (A) Structure of lovastatin and (B) mechanism of action of lovastatin](image-url)
Strain improvement of *Aspergillus terreus* for hyper-production of lovastatin

incubator and counted the fungal colonies in each plate (10). The mutants were randomly selected from the plates having at least 80-85% death rate or 15-20% survivors, especially those which showed morphological changes. Mutants were transferred to fresh slants and incubated at 30°C for 6 days. All the selected mutants were tested for lovastatin production.

**Analysis of lovastatin**

The identifications and quantifications of lovastatin were carried out on the culture filtrate by HPLC method (11). Fungus secretes the lovastatin in its β-hydroxyacid form which are quite stable form in solution and eluted first from column than lactone form of the drug. The filtered broth was diluted 10-fold with acetonitrile-water (1 : 1 by volume). The samples were analyzed by using an HPLC (Hitachi) with UV detector (Hitachi L-2400) at 238 nm and Hitachi L-2130 (C-18) column. The solvent was a mixture of acetonitrile and 0.1% phosphoric acid (60 : 40 by volume) with flow rate of 1.5 mL/min. The sample injection volume was 20 µL.

**Optimization of fermentation parameters**

Different parameters were optimized for production of lovastatin by *Aspergillus terreus* S-24

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**Table 1. Effect of UV radiation dose to formulate the kill curve.**

<table>
<thead>
<tr>
<th>UV treatment time (min)</th>
<th>CFU/mL</th>
<th>Ln CFU/mL</th>
<th>%age of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15×10^9</td>
<td>25.73</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>7×10^9</td>
<td>22.66</td>
<td>88.06</td>
</tr>
<tr>
<td>40</td>
<td>13×10^9</td>
<td>20.98</td>
<td>81.53</td>
</tr>
<tr>
<td>60</td>
<td>14×10^9</td>
<td>18.75</td>
<td>72.87</td>
</tr>
<tr>
<td>80</td>
<td>18×10^9</td>
<td>16.70</td>
<td>64.9</td>
</tr>
<tr>
<td>100</td>
<td>8×10^9</td>
<td>13.59</td>
<td>52.8</td>
</tr>
<tr>
<td>120</td>
<td>7×10^9</td>
<td>11.15</td>
<td>43.33</td>
</tr>
<tr>
<td>140</td>
<td>9×10^9</td>
<td>9.1</td>
<td>35.3</td>
</tr>
<tr>
<td>160</td>
<td>2×10^9</td>
<td>5.29</td>
<td>20.5</td>
</tr>
<tr>
<td>180</td>
<td>5×10^9</td>
<td>3.91</td>
<td>15.15</td>
</tr>
<tr>
<td>210</td>
<td>2</td>
<td>0.69</td>
<td>2.68</td>
</tr>
</tbody>
</table>

Values (Mean ± SD) are average of three samples; CFU: Colony forming units/mL; %age of survival = (Colony count after exposure time t) ×100/Total colonies; %age of killing = 100 - %age of survival

**Table 2. Estimation of lovastatin production by parent and mutant *Aspergillus terreus* strain.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dry cell mass (mg/mL)</th>
<th>Lovastatin (mg/100 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>5.37 ± 1.25</td>
<td>16.74 ± 1.09</td>
</tr>
<tr>
<td>ATU-01</td>
<td>4.22 ± 0.61</td>
<td>27.23 ± 1.81</td>
</tr>
<tr>
<td>ATU-02</td>
<td>4.15 ± 0.14</td>
<td>31.87 ± 0.76</td>
</tr>
<tr>
<td>ATU-03</td>
<td>5.16 ± 0.13</td>
<td>37.54 ± 1.01</td>
</tr>
<tr>
<td>ATU-04</td>
<td>5.29 ± 1.12</td>
<td>39.77 ± 0.61</td>
</tr>
<tr>
<td>ATU-05</td>
<td>4.28 ± 1.31</td>
<td>29.65 ± 1.11</td>
</tr>
<tr>
<td>ATU-06</td>
<td>5.37 ± 0.29</td>
<td>51.66 ± 0.88</td>
</tr>
<tr>
<td>ATU-07</td>
<td>4.17 ± 1.31</td>
<td>25.62 ± 1.31</td>
</tr>
<tr>
<td>ATU-08</td>
<td>3.27 ± 1.29</td>
<td>43.58 ± 0.83</td>
</tr>
<tr>
<td>ATU-09</td>
<td>4.14 ± 1.41</td>
<td>34.35 ± 0.51</td>
</tr>
<tr>
<td>ATU-10</td>
<td>6.64 ± 0.61</td>
<td>44.64 ± 1.19</td>
</tr>
</tbody>
</table>

Values (Mean ± SD) are average of three samples, Analyzed individually in triplicate (n = 1 × 3 × 3) p ≤ 0.05; Vogel medium (KH₂PO₄, 0.5; peptone, 0.1; yeast extract, 0.2, NH₄NO₃, 0.2; (NH₄)₂SO₄, 0.4; MgSO₄, 0.02; glucose, 50%; trisodium citrate, 0.5g/100 mL); ATU, UV treated *Aspergillus terreus*; Temperature, 30°C, Incubation time 48 h
parent and *Aspergillus terreus* ATU-06 mutant strain. The Vogel medium was fermented in triplicate in flasks with *Aspergillus flavus* containing selected substrate wheat bran at varying pH, temperature, inoculum size, moisture contents, fermentation time and nitrogen sources.

**Immobilization of mutant ATU-06**

All the immobilization processes were performed under aseptic conditions. The cell pellet of *A. terreus* ATU-06 was obtained in logarithmic phase of growth was collected by centrifugation at 5000 rpm in centrifuge (Mikro 20 Hettich, Germany). 0.38 g fungal cells were obtained from culture mixed with 10 mL sodium alginate solution at a concentration of 3% (w/v) through a sterile pipette. Beads of 2 mm diameter were used for entrapment in agar (0.38 g) and agarose (0.6 g) wet cells were mixed with 10 mL of 3% agar solution at 45°C. The mixture was allowed to quickly cool at 4°C, cut into 2 × 2 × 2 mm fragments and transferred to 25 mL of the production medium.

**Repeated batch experiment**

The experimental set up was similar to the process described above. Every 96 h, immobilized cells were removed, washed with saline and recultivated into fresh medium. The process was repeated for several batches till the beads started disintegrated.

**Statistical analysis**

Statistical significance of the differences between mean values was assessed two way analysis of ANOVA under CRD and DMR test using Minitab 2000 version 13.2 statistical software (Minitab Inc., Pennsylvania, USA). A probability value of \( p \leq 0.05 \) was considered to denote a statistically significant difference (13).

**RESULTS**

**Induction of mutation**

In present investigation, efforts have been made to improve the native *Aspergillus terreus* strain by UV irradiation for enhanced production of lovastatin. In the case of UV irradiation treatment above 15.5% survivor rate was obtained after 180 min, which decreased gradually with the increase of exposure time (Table 1). These mutants were further evaluated through secondary metabolite production by solid state fermentation. The selected 10 mutants from UV mutants of *Aspergillus terreus* were used for the production of lovastatin (selected on the basis of their ability to produce maximum lovastatin), using bagasse for the selection of best mutant. Among theses, the maximum lovastatin production (51.66 ± 0.88 mg/100 mL) with dry cell mass (6.64 ± 0.61 g/L) was observed for the mutant ATU-06 using bagasse (Table 2). The results indicated ATU-06 was the most hyper-active mutant, giving approximately 5.67 fold more lovastatin over the parent strain (16.74 ± 1.09 mg/100 mL) in similar culture conditions.

**Kinetic parameters**

Comparison of lovastatin production by parent *Aspergillus terreus* S-24 strain and mutant ATU-06 strain is presented in Table 3. The parent and mutant strain of *Aspergillus terreus* showed maximum lovastatin production 12.74 ±1.09 mg/100 mL and 28.31±0.96 mg/100 mL before optimization of environmental factors and it was further improved to 27.66 ± 0.88 mg/100 mL and 87.33 ± 1.94 mg/100 mL, respectively, on post optimization. The growth parameters for pre and post optimization of growth yield coefficients, volumetric rates and specific sub-

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Aspergillus terreus</th>
<th>Cell biomass (g/L)</th>
<th>Sugar consumed (g/L)</th>
<th>Lovastatin (mg/100 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Parent</td>
<td>5.71 ± 1.21</td>
<td>30.1 ± 1.86</td>
<td>12.74 ± 1.09</td>
</tr>
<tr>
<td>Optimization</td>
<td>Mutant</td>
<td>4.22 ± 1.01</td>
<td>15.21 ± 0.85</td>
<td>28.31 ± 0.96</td>
</tr>
<tr>
<td>Post</td>
<td>Parent</td>
<td>5.42 ± 0.52</td>
<td>25.7 ± 1.84</td>
<td>27.66 ± 0.88</td>
</tr>
<tr>
<td>Optimization</td>
<td>Mutant</td>
<td>3.67 ± 0.41</td>
<td>7.28 ± 1.92</td>
<td>87.33 ± 1.94</td>
</tr>
</tbody>
</table>

Optimum pH (6), temperature 30°C, inoculum size 2 mL, moisture contents 60% and incubation time 120 h for both parent and mutant strain; Values (Mean ± SD) are average of three samples, Analyzed individually in triplicate (n = 1 × 3 × 3) p ≤ 0.05; Lovastatin was produced in basal medium containing initial sugar concentration 50 g/L (w/v).
Strain improvement of Aspergillus terreus for hyper-production of lovastatin rates were also studied. The mutant strain of Aspergillus terreus ATU-06 significantly improved the values of Yx/s (drug produced/g glucose consumed), Yp/x (drug produced/g cell formed) and Yp/s (Cell formed/g sugar consumed) (1.86 mg/g, 5.42 mg/g and 3.66 mg/g/h) over the parental strain Aspergillus terreus (0.42 mg/g, 2.23 mg/g and 5.27 mg/g/h) before optimization, and on optimization it was observed that these growth parameters were further improved by parent (1.07 mg/g, 2.23 mg/g and 5.09 mg/g/h) as well as mutant strain (11.99 mg/g, 23.79 mg/g and 1.98 mg/g/h), respectively (Table 4).

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Aspergillus terreus</th>
<th>Pre optimization</th>
<th>Post optimization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent ATU-06</td>
<td>Parent ATU-06</td>
<td>Parent ATU-06</td>
</tr>
<tr>
<td>Aµ (h⁻¹)</td>
<td>0.105</td>
<td>0.156</td>
<td>0.141</td>
</tr>
<tr>
<td>oYx/s</td>
<td>0.42</td>
<td>1.86</td>
<td>1.07</td>
</tr>
<tr>
<td>oYp/x</td>
<td>2.23</td>
<td>5.42</td>
<td>9.53</td>
</tr>
<tr>
<td>oqs (g/g/h)</td>
<td>5.27</td>
<td>3.60</td>
<td>5.09</td>
</tr>
<tr>
<td>oQs (g/L/h)</td>
<td>1.806</td>
<td>0.9126</td>
<td>1.542</td>
</tr>
<tr>
<td>oYp/s</td>
<td>0.342</td>
<td>0.25</td>
<td>0.32</td>
</tr>
<tr>
<td>oQp (Product/L/h)</td>
<td>0.189</td>
<td>0.277</td>
<td>0.21</td>
</tr>
<tr>
<td>oQp (Product/h/g)</td>
<td>1.21</td>
<td>1.99</td>
<td>2.49</td>
</tr>
<tr>
<td>oQp (Product/L/h)</td>
<td>0.7644</td>
<td>1.69</td>
<td>3.09</td>
</tr>
</tbody>
</table>

Product was produced in basal medium, initial sugar concentration was 50 g/L (w/v); Aµ (h⁻¹), Specific growth rate; oYx/s, drug produced/g glucose consumed; oYp/x, drug produced/g cell formed; oqs (g/g/h), g sugar consumed/g cells/h; oQs (g/L/h), g sugar consumed/L/h; oQp (g/L/h), g cells formed/L/h; oYp/s, Cell formed/g sugar consumed; oQp (g/g/h), drug produced/h/g cells; oQp (g/L/h), drug produced/L/h

Immobilization of Aspergillus terreus ATU-06 mutant

The best mutant Aspergillus terreus ATU-06 was immobilized on calcium alginate and agar by entrapment method. It was obtained that effective-
ness of the immobilization was less than one (0.683, 0.689 and 0.72 for agar, agarose and calcium alginate immobilized cells, respectively) and the activity of immobilized cells was lower than free cells (Table 5). On comparison of both carriers for immobilization it was concluded that calcium alginate beads exhibited 0.72 effectiveness factor higher than agar and agarose (i.e. 0.683 and 0.689).

The possibility of repeated use of immobilized cells of *Aspergillus terreus* was studied. It was found that multiple uses of immobilized cells were possible as compared to free cells. Results have shown in Figure 2 indicated that free cells could not retain their ability to produce lovastatin consistently after 2 cycles with residual activity of 47.90% while immobilized cells showed the ability to produce lovastatin after five cycles with 62.9% residual activity. This loss in activity was due to destruction of cells as well as autolysis during the centrifugation and washing process.

**DISCUSSION**

Industrial strain improvement plays a key role in the development of microbial fermentation process commercially. The improvement of lovastatin producing strain has been carried out by mutagenesis and selection. The most employed technique has been the induction of mutation in parental strain by mutagens (9). The mutants produced as a result of induced mutation are used for commercial secondary metabolite production (10). Among all mutagens, UV radiation, chemical mutagens and gamma radiations are often used. In present study, strain of *Aspergillus terreus* S-24 was improved by UV irradiation for hyper production of lovastatin (Table 1 and 2). Similar trend of decrease in survivability with increase in exposure time has also been reported by some other investigators (14). Genetic changes in organisms by UV irradiation increase the yields of certain chemicals by the organisms. Samiee et al. (15) found *Aspergillus terreus* as a best lovastatin (55 mg/L) producing strain out of 110 fungal strains including some selected strains. Mukhtar et al. (14) reported the mutant strain UV-4 gave the maximum lovastatin production (1396.09 ± 0.08 µg/mL) with a dry cell mass of 19.47 ± 0.11 mg/mL. All these findings indicated that the survivability of parent strain depended on the nature of the microorganism, treatment period and the type of mutagens.

Optimization of environmental factors for maximum production of secondary metabolites is very important. The findings of Mukhtar et al., (14) were in accordance to our results. They also reported the increase in lovastatin production on optimization of fermentation conditions by *Aspergillus terreus* NRRL-265 mutant strain. Potential kinetic parameters for production of lovastatin by *Aspergillus terreus* S-24 and mutant *Aspergillus terreus* ATU-06 during growth in optimized media in solid state fermentation are in Table 3 and 4. Iftikhar et al. (16) observed Yp/x and Yp/s 1040 IU/g and 2080 IU/g in shake flask while 1650 IU/g and 2750 IU/g in fermentor during study of production of lipase by hyper producing mutant of *Rhizopus oligosporus GDM: 31*. Nadeem et al. (17) reported that maximum Yp/x and Yp/s were 264.28 PU/g of cell biomass and 99.3 PU/g of glucose, respectively, after incubation and maximum cell growth rate 0.308 by *B. licheniformis N-2* strain after 8 h of inoculation. He et al. (18) found maximum growth rate of 0.17 by *Bacillus sp* after 6 h of inoculation. A maximum growth rate of 0.7, biomass (0.023g of cells/g of glucose) and product yield (0.0211 U/g) for *Bacillus L21* was reported by Genckal et al. (19).

On immobilization of *Aspergillus terreus* ATU-06 mutant strain it was evident that reuse of strain could be possible (Table 5 and Fig. 2) Our results were in close agreement to the Ahmad and Abdel-Fattah (20) findings who also reported lower activity of the immobilized cells when they immobilized...
bacterial cells on different carriers by different method. Less accessibility of nutrients to immobilized cells and low availability of oxygen can cause decreased activity of immobilized cells. Immobilization also changes the metabolic and morphological changes in the cells. Farag et al. (21) reported that *Aspergillus terreus* cells are reused for eight successive cycles but the activity increased slightly up to four cycles then the reuse culture exhibited a gradual decrease up to eight run. Wool immobilized *Bacillus licheniformis* ATTC 21415 was useful for 5 batches reported by Ahmed and Abdel-Fattah (20).

**CONCLUSION**

It was concluded from above study that *Aspergillus terreus* ATU-06 showed better results for the production of lovastatin and immobilized cells were more efficient for lovastatin production than free cells for repeated use. The present data would certainly help to ascertain the ability of ATU-06 mutant as potential source of hypocholesterolemic molecule lovastatin to be used in clinical treatments.

**Conflict of interest**

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Acknowledgment**

The data of the paper are the part of the M. Phil. thesis of second author (Amair Raza).

**REFERENCES**


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Glucotoxicity, lipotoxicity, and glucolipotoxicity are secondary phenomena that are proposed to play a role in all forms of type 2 diabetes. The underlying concept is that once the primary pathogenesis of diabetes is established, involving probably both genetic and environmental forces, hyperglycemia and very commonly hyperlipidemia ensue and thereafter exert additional damaging or toxic effects on the β cells. Elevations of plasma fatty acid levels that often accompanying insulin resistance (1, 2) may also play a pathogenic role in the early stages of the disease as glucose levels begin to rise outside of the normal range, as well as their contribution on the deterioration of β cell function after the onset of the disease (3).

The abnormal metabolic medium created by hyperglycemia or the diabetic state initiates series of dysfunctional responses culminating in the development of premature cardiovascular disease (4, 5). The oxidative stress pathway serves, as a common element, to link all the major pathways implicated in diabetic vasculopathy (6). Hyperglycemia induces oxidative stress in vascular cells by enhancing the production of reactive oxygen species (ROS). These results in damage to cellular proteins, membrane lipids, DNA and activation of nuclear factor-κB (NF-κB) (5).

Fruits and vegetables are primarily food sources providing essential nutrients for sustaining life. They also contain a variety of phytochemicals such as phenolics and flavonoids providing important health benefits. Thus, regular consumption of fruits and vegetables is associated to reduced risks of chronic diseases, such as cancers and cardiovascular disease (7).
Brassica Rapa (Br) has active biological compounds such as flavonoids (isorhamnetin, kaempferol and quercetin glycosides), phenylpropanoids derivatives (8), indole alkaloids and sterol glucosides (9). Indole fraction from Brassica rapa roots is known to possess anti-inflammatory potential via inhibition of pro inflammatory mediators such TNFα and interleukin-6 (10), antidiabetic effect in diabetes mellitus type 2 (11, 12). We found out that it was interesting to induce a glucolipotoxicity in vivo by a high fat high sucrose diet, in Psammomys obesus and analyze the Brassica rapa therapeutic effect.

EXPERIMENTAL

Biological material

The present study was approved by the Institutional Animal Care and Use Committee of the University of Bab Ezzouar (Algiers, Algeria; Permit number for the present research project: F00220110048) and has been achieved according to the Executive Decree no. 10ñ90 completing the Executive Decree no. 04ñ82 of the Algerian Government, establishing the terms and modalities of animal welfare in animal facilities.

Psammomys obesus gerbils also known as the “fat sand rat” were captured in the Algerian Sahara. During two week acclimation period, the animals were fed with the halophilic plants, rich in water and mineral salts (13), that they normally eat in the desert. They were put in individual cages under controlled temperature and lighting conditions, with free access to food and water. They were divided into four groups. The first group (n = 8 normal control), the second group (n = 8 normal treated with Br 200 mg/kg during 20 days by oral gavage) were fed, during 9 months, by natural plants from the same halophilic family during 9 months, but growing along the edge of sea (salicornia; composition per 100 g: water 80.8 g; mineral salts 6.9 g; lipids 0.4 g; proteins 3 g; carbohydrates 8.4 g; and 45-50 kcal/100 g). The third group (n = 8 diabetic control) and the fourth group (n = 8 diabetic treated with Br 200 mg/kg during 20 days by oral gavage) received during 6 months a high-fat (10%) and high sucrose (20%) diet, comprising the salicornia plants plus the daily addition of half of cooked egg yolk and 20 g of saccharose per day (composition of egg per 100 g: water 40-60 g; proteins 13.5 – 17.5 g; carbohydrates 0.2 g; lipids 30 – 31 g; cholesterol 1.2 – 1.3 g; and 370 – 400 kcal/100g).

Preparation of aqueous extract of Brassica rapa var rapiferra

Fresh roots of B. rapa var. rapiferra were collected from Algeria’s markets in February 2012. Voucher specimen (INA/P/No 6) has been kept in the herbarium of the Department of Botany, National Institute of Agronomy (INA), Algiers, Algeria. The roots were separated from turnip tops, each cut into small slices and dried in shade till complete drying. The plant material was pulverized into powdered form. The aqueous extract was prepared by decocting powdered roots (100 g) three times till complete exhaustion, then the collected aqueous extract was lyophilized (Cryodos 80, -75OC, 5 m3/h) to obtain extract in yield of 0.15%. The extract was stored in sealed glass vials at ± 4°C prior to be tested and analyzed.

Chemical study

Total phenolic content

Total phenolic contents of the extract were determined using Folin-Ciocalteu reagent according to the method of (14), using gallic acid as a standard, and as modified by (15). An aliquot (0.2 mL) of extract solution containing 1000 µg of extract was mixed with 46 mL of distilled water and 1 mL of Folin-Ciocalteu reagent in a volumetric flask. After spending 3 min in the dark, 3 mL of sodium carbonate solution (7.5%) was added. Absorbance at 740 nm was measured in a spectrophotometer (Shimadzu 1800, Mulgrave, Victoria, Australia) after shaking and spending an additional 2 h in the dark. The total phenolic content was evaluated from a standard calibration curve of gallic acid, and results were expressed as microgram of gallic acid equivalents per milligram of extract (µGAE/mg).

Table 1. Total phenolic and flavonoid contents and antioxidant activity of Brassica rapa var. rapiferra (Br).

<table>
<thead>
<tr>
<th>Extract / Standards</th>
<th>Total phenolic content (µg GAE/mg)</th>
<th>Total flavonoids (µg QE/mg)</th>
<th>DPPH (IC₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>9.41 ± 0.18</td>
<td>1.01 ± 0.09</td>
<td>2100 ± 13</td>
</tr>
<tr>
<td>BHA</td>
<td>n.a</td>
<td>n.a</td>
<td>21.28 ± 0.12</td>
</tr>
<tr>
<td>BHT</td>
<td>n.a</td>
<td>n.a</td>
<td>12.76 ± 0.08</td>
</tr>
</tbody>
</table>

Each value is expressed as the means ± standard deviations for triplicate experiments. n.a: not applied.
Therapeutic effect of *Brassica rapa var rapifera* in type...

**Determination of total flavonoids**

The total flavonoids were determined according to the Dowd method described by (15). 4 mL of diluted solution of extract was mixed with 4 mL of aluminum trichloride solution (2% in methanol). After 15 min, the absorbance was measured at 415 nm. Quercetin was used as reference compound to produce the standard curve. The results are expressed as µgQE/mg.

**Antioxidant activity: Scavenging effect on DPPH radical**

The DPPH free radical scavenging assay was carried out as described by (16). The aqueous extract was dissolved in methanol. Sample of 25 µL of each concentration (100, 200, 400, 600, 800 and 1000 µg/mL) were added to the DPPH methanol solution (60 µM, 975 µL). After 30 min of incubation at 25°C, the absorbance at 517 nm was measured by using UV spectrophotometer (Jasco, V-530). Ascorbic acid and α-tocopherol were used as reference compounds. The radical scavenging activity was then calculated from the equation: % of radical scavenging activity = [(Abscontrol − Absy)/Abscontrol] × 100, where Abscontrol is the absorption of the blank sample and Abssample is the absorbance of the tested extract.

**Biological study**

**Analytical methods**

The animals were bled from the retro-orbital venous plexus; this technique eliminates the use of anesthetic agents affecting measurements of biochemical parameters. Blood, collected in dries tubes, was centrifuged at 3000 rpm for 10 min and stored at -30°C. Blood glucose, triglyceride, cholesterol and protein were measured by enzymatic colorimetric method using a test kit of Biosystem. Blood insulin was determined, by radioimmunoassay, using CIS test kit (ORIS INDUS) CPK. In addition, uric acid was determined by the automate CKL, 0-323.

**Isolation of tissue**

At the end of the experiment, animals were killed after anesthesia with urethane (25%). The aorta and heart tissues were immediately excised, frozen in liquid azote and stored at -80°C. The tissue was extracted in ice-cold solubilization buffer by using a motor-driven Potter homogenizer (20 mM/L HEPES, 8 mM/L EDTA, 0.2 mM/L Na3VO4, 10 mM/L Na2P04, 2.5 mM/L phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, 2.5 mg/mL benzamidine, 2.5 mg/mL pepstatin, 2.5 mg/mL leupeptin, 160 mM/L NaF, 2 mM/L dichloroacetic acid, 1% Triton X-100, pH 7.4. After 20 min at 4°C, the samples were centrifuged at 20000 × g, the supernatants were stored at -80°C.

**Determination of malondialdehyde (MDA)**

The MDA was evaluated after reaction with thiobarbituric acid (TBA) (Sigma) (17). The samples were centrifuged at 10000 × g for 20 min at 4°C in buffered (Na2HPO4/NaH2PO4) 0.2 M, pH 6.5. The MDA contained in the supernatant in the presence of 10% TCA reacts with TBA and causes the formation of a complex read at 532 nm.

**Catalase activity determination (CAT)**

CAT activity was determined by monitoring the disappearance of H2O2 at 240 nm. The reaction mixture contained 50 mM K-phosphate buffer (pH 7) 0.33 mM H2O2 and enzyme extract (18).

**Measurement of inflammation insulin pathway markers**

The assessment was determined by immunoenzymatic assay. Invitrogen ELISA Kits were used for measuring the levels of different markers in the tissue. IRS 1 [S312], AKT [S p 473], and NF-κB p65. The estimation is made by Elisa reader at 450 nm (BioTek Instruments).

**Statistical analysis**

Data were analyzed with ANOVA using STATISTICA version 6 and completed with HSD Tukey test. The results were expressed as the mean ± standard deviation.

The differences at level p < 0.05 were considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Chemical study**

**Total phenolics and flavonoids contents**

The aqueous extract showed a weak amount of phenolic and flavonoid compounds with values of 9.41 ± 0.18 µg GAE/mg and 1.01 ± 0.09 µg QE/mg, respectively (Table 1).

**Antioxidant activity**

The principle antioxidant activity was based on the availability of electrons to neutralize any free radicals. In the present study, the antioxidant activity was evaluated using scavenging DPPH free radicals assay. The results of the aqueous extract and those controls (BHA and BHT).

The investigated aqueous extract of *Brassica rapa* showed a weak antiradical activity with IC50...
value of 2100 ± 13 µg/mL, which was extremely lower than the high antioxidant effect of BHA and BHT (IC₅₀ = 21.28 ± 0.12 µg/mL and 12.76 ± 0.08 µg/mL, respectively) (Table 1).

**Effect of Br on biochemical parameters of Po**

As concerns animals submitted to natural diet supplemented with high fat-sucrose diet (P diabetic) during 9 months of experiment, we noted a high increase of serum glucose, proteins and dyslipidemia which was marked by TC, TG, VLDL, LDL and decreased HDL in P diabetic. Therefore, a highly significant increase of cardiac marker CPK and LDH and kidney marker creatinine were observed in P diabetic rats compared to P control. The treatment with aqueous extract of *Brassica rapa* (Br) ameliorated the deregulation observed under a high fat high sucrose diet (Table 2a).

**Effect of Br redox statues of Po**

The elevation of plasma and erythrocyte MDA level in the group of P diabetic was highly significant but reversed with the Cat in this condition. The treatment with of Br prevented this oxidative stress (Table 2b).

The evaluation of insulinemia showed an increase after 3 months and a decrease after 9 month of experiment (Fig. 1).

**Effect of Br on inflammatory parameters and redox statues of Po**

In both aorta and heart tissue, the AKT level was decreased while the NFκB level was increased in P diabetic. The treatment with Br modulated these parameters. The evaluation of redox status parameters showed an increase of MDA and a decrease of Cat levels in aorta and heart of P diabetic compared to P control, and reversed with treatment of Br in P diabetic +Br (Table 3 and 4).

This study emphasized on the protective effect in vivo of aqueous extract of *Brassica rapa* (Br) in a high fat-sucrose diet treated diabetic rats.

Total phenolics and flavonoids contents were determined according to their importance as an

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P control</th>
<th>P control + Br</th>
<th>P diabetic</th>
<th>P diabetic + Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (g/L)</td>
<td>0.65 ± 0.05</td>
<td>0.62 ± 0.06</td>
<td>1.81 ± 0.12****</td>
<td>1.15 ± 0.13++</td>
</tr>
<tr>
<td>Triglyceride (TG) (g/L)</td>
<td>0.38 ± 0.08</td>
<td>0.37 ± 0.09</td>
<td>3.46 ± 167****</td>
<td>1.68 ± 0.4++</td>
</tr>
<tr>
<td>Cholesterol total (TC)(g/L)</td>
<td>0.9 ± 0.06</td>
<td>0.81 ± 0.02</td>
<td>4.88 ± 0.22****</td>
<td>2.35 ± 0.53++</td>
</tr>
<tr>
<td>LDL-chol. (mM/L)</td>
<td>0.54 ± 0.26</td>
<td>0.49 ± 0.01</td>
<td>27.55 ± 3.18****</td>
<td>0.64 ± 0.14++++</td>
</tr>
<tr>
<td>HDL-chol. (mM/L)</td>
<td>1.89 ± 0.21</td>
<td>1.71 ± 0.1</td>
<td>1.05 ± 0.08**</td>
<td>5.37 ± 0.67++++</td>
</tr>
<tr>
<td>Creatinine (U/L)</td>
<td>28 ± 1.41</td>
<td>25 ± 0.01</td>
<td>114 ± 17.61****</td>
<td>44.75 ± 14.08+++</td>
</tr>
<tr>
<td>CPK (U/L)</td>
<td>4 ± 0.1</td>
<td>4 ± 0.5</td>
<td>642.5 ± 114.49****</td>
<td>90.75 ± 7.41++++</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>4 ± 2.82</td>
<td>3 ± 1.13</td>
<td>92 ± 10.10****</td>
<td>13.75 ± 4.27+++</td>
</tr>
<tr>
<td>Protein (mg/dL)</td>
<td>0.67 ± 0.03</td>
<td>0.66 ± 0.06</td>
<td>0.87 ± 0.00**</td>
<td>0.66 ± 0.03++</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.E.M; (n = 8). **p < 0.001, ****p < 0.0001 (P diabetic vs P control), ++p < 0.001, ++++p < 0.0001 (P diabetic + Br vs. P diabetic); P control: *Psammomys obesus* control; P control + Br: *Psammomys obesus* control treated with extract aqueous of *Brassica rapa*; P diabetic: *Psammomys obesus* received a high-fat (10%) and high sucrose (20%) diet; P diabetic + Br: *Psammomys obesus* received a high-fat-sucrose diet and treated with extract aqueous of *Brassica rapa*.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P control</th>
<th>P control + Br</th>
<th>P diabetic</th>
<th>P diabetic + Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (U/L)</td>
<td>0.17 ± 0.01</td>
<td>0.35 ± 0.03</td>
<td>0.07 ± 0.00****</td>
<td>0.61 ± 0.01++</td>
</tr>
<tr>
<td>Catalase eryth. (U/L)</td>
<td>1.43 ± 0.26</td>
<td>2.19 ± 0.08</td>
<td>1.20 ± 0.08</td>
<td>2.36 ± 0.64++++</td>
</tr>
<tr>
<td>MDA (µM/L)</td>
<td>72.8 ± 5.31</td>
<td>71.2 ± 3.65</td>
<td>149.8 ± 2.60****</td>
<td>71.4 ± 0.89++++</td>
</tr>
<tr>
<td>MDA eryth (µM/L)</td>
<td>87 ± 1.41</td>
<td>80 ± 0.56</td>
<td>108 ± 1.58****</td>
<td>92.2 ± 3.96+++</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.E.M; (n = 8). **p < 0.001, ****p < 0.0001 (P diabetic vs P control), ++p < 0.001, ++++p < 0.0001 (P diabetic + Br vs P diabetic). Eryth.: erythrocyte.
therapeutic effect of *Brassica rapa var rapifera* in type 2 diabetes.

Antioxidant compounds. In fact, there is a relationship between the antioxidant ability and the total phenol contents. Phenolic antioxidants are products of a secondary metabolism in plants, and the antioxidant activity is mainly induced in their redox properties and chemical structure, which can play an important role in chelating transitional metals, inhibiting lipoxygenase and scavenging free radicals (19). The principle antioxidant activity is based on the availability of electrons to neutralize any free radicals. In the present study, the antioxidant activity was evaluated by determining IC_{50}, using scavenging DPPH free radicals. The value of IC_{50} was used in the *in vitro* study. This activity could be

![Figure 1. Effect of aqueous extract of Br in insulinemia after the administration of high fat-sucrose diet in po. Data are expressed as the mean ± S.E.M; (n = 8). ****p < 0.0001, **p < 0.01 (P diabetic vs. P control), NS, ++++p < 0.0001 (P diabetic + Br vs. P diabetic).](image)

Table 3. Effect of aqueous extract of *Br* in the inflammation-oxidative stress parameters of the aorta after the administration of high fat-sucrose diet in *Po*.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P control</th>
<th>P control + Br</th>
<th>P diabetic</th>
<th>P diabetic + Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (pM/100 g)</td>
<td>2.68 ± 2.27</td>
<td>2.65 ± 2.00</td>
<td>179.75 ± 44.8****</td>
<td>3.03 ± 1.04++++</td>
</tr>
<tr>
<td>Catalase(UI/min/g protein/100 g)</td>
<td>35.93 ± 5.74</td>
<td>33.8 ± 2.6</td>
<td>6.74 ± 139****</td>
<td>24.38 ± 8.10++++</td>
</tr>
<tr>
<td>AKT (pg/mL)</td>
<td>1.15 ± 0.53</td>
<td>1.04 ± 0.43</td>
<td>0.41 ± 0.29****</td>
<td>1.01 ± 0.04++++</td>
</tr>
<tr>
<td>NFκB (ng/mL)</td>
<td>9.80 ± 0.63</td>
<td>9.76 ± 0.54</td>
<td>92.70 ± 0.63****</td>
<td>16.3 ± 0.59++++</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.E.M; (n = 8). ****p < 0.0001 (P diabetic vs. P control), ++++p < 0.0001 (P diabetic + Br vs. P diabetic)

Table 4. Effect of aqueous extract of *Br* in the inflammation-oxidative stress parameters of the heart after the administration of high fat-sucrose diet in *Po*.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P control</th>
<th>P control + Br</th>
<th>P diabetic</th>
<th>P diabetic + Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (pM/100 g)</td>
<td>1.21 ± 0.27</td>
<td>1.18 ± 0.10</td>
<td>1.98 ± 0.08**</td>
<td>0.89 ± 0.20++</td>
</tr>
<tr>
<td>Catalase(UI/min/g protein/100 g)</td>
<td>17.63 ± 11.52</td>
<td>16.33 ± 0.69</td>
<td>10.72 ± 3.53**</td>
<td>30.80 ± 24.46</td>
</tr>
<tr>
<td>AKT (pg/mL)</td>
<td>0.15 ± 0.03</td>
<td>0.14 ± 0.02</td>
<td>0.01 ± 0.00****</td>
<td>0.16 ± 0.01++</td>
</tr>
<tr>
<td>NFκB (ng/mL)</td>
<td>4.22 ± 1.32</td>
<td>4.20 ± 8.84</td>
<td>5.92 ± 0.08</td>
<td>4.06 ± 1.78</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.E.M; (n = 8). **p < 0.001, ****p < 0.0001 (P diabetic vs. P control), ++p < 0.001, (P diabetic + Br vs. P diabetic)
explained by the presence of the phenolics compounds which are related to different mechanisms, such as free radical-scavenging, hydrogen-donation, singlet oxygen quenching, metal ion-chelation, and acting as substrates for radicals such as superoxide and hydroxyl (20).

In our study, we found a hyperglycemia associated to hypertriglyceridemia and hypercholesterolemia characteristic of type 2 diabetes (21-23). After 20 days of treatment with aqueous extract of Brassica rapa (200 mg/kg) ameliorated this biochemical parameters in diabetic rats. Our results were according to (24) in alloxan diabetic rats and treated with Brassica rapa (200 mg/kg), in streptozotocin diabetic rats and treated with different dose of Brassica nigra (25) and in diabetic rats treated with Brassica oleracea (26).

The hypoglycemic effect of the seed extract of Brassica juncea was attributed to the stimulation of glycogen synthesis leading the increase of hepatic glycogen content and the suppression of glycogen phosphorylase and other glyconeogenic enzymes activity (27). Of the same, oral administration of BNO (Brassica Nigra Oil) 1000 mg/kg body weight significantly increased the hepatic glycogen levels in STZ diabetic rats, possibly because of reactivating the glycogen synthase system as a result of the presence of H2O2 (41, 42). There is a clear link triggering the production of peroxides in the medium in presence of BNO, which may result an inhibition of lipolysis and increased plasma insulin concentrations in diabetic rats. In (28) it was demonstrated that BNO (Brassica nigra) 1000 mg/kg body weight was effective in reducing the incidence of diseases risks (45) suggested that impaired blood lipids were the characteristic of subjects with insulin resistance, especially circulating FFAs (46). Fatty acid mobilization from adipose tissue is sensitive to insulin. Insulin is most potent acting in the suppression of adipose tissue lipolysis. A rise of insulin resistance, especially circulating FFAs (46). Low levels of lipid peroxides stimulate the secretion of insulin but when the concentration of endogenous peroxides increases, it may initiate uncontrolled lipid peroxidation, thus leading to cellular infiltration and islet cell damage in type I diabetes (36).

The increased lipid peroxidation in the tissues of diabetic animals may be induced by the observed increase in the concentration of TBARS in the liver and kidney of diabetic rats (37).

Catalase is a hem-protein which is responsible for the detoxification of significant amounts of H2O2 (38). Reduced activities of catalase in the liver and pancreas during diabetes were reported, resulting in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (39). CAT protects tissues from extreme toxic hydroxyl through decomposing of hydrogen peroxide (40). A dual action of polyphenols as both antioxidant and prooxidant, has been demonstrated in cell culture systems with the common plant polyphenols, quercetin and epigallocatechin-3-gal late. These polyphenols have been shown to decrease production of ROS in the cells but also trigger the production of peroxides in the medium in the presence of H2O2 (41, 42). There is a clear link between hyperglycemia and active oxygen/nitrogen species in experimental and clinical types of diabetes (43). Accumulation of reactive oxygen species (ROS) due to oxidative stress is also instrumental in the expression of cell death as ROS can easily react with and oxidize vital cellular components such as lipids, proteins and DNA (44).

In our study, we showed a compensatory hyperinsulinemia associated with hyperlipidemia after 3 months of a high fat-sucrose diet indicator of insulin resistance. In (28) it was demonstrated that BNO increased plasma insulin concentrations in diabetic rats. Insulin levels higher than those of the control group may result an inhibition of lipolysis and decreased plasma triglyceride and cholesterol levels. These observations were supported by a recent evidence that FFAs activate directly macropage to secrete pro-inflammatory cytokines that render muscle cells insulin resistant (48). Meanwhile, the role...
of pro-inflammatory cytokines in regulating insulin sensitivity has been suggested by several lines of evidence. To this effect, subjects with T2DM exhibited higher serum levels of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 (49). Moreover, FFAs contribute on the increased production of reactive oxygen species and lead to the activation of stress-sensitive signaling pathways under hyperglycemic status (50). The polyphenols of quercetin had anti-oxidative and anti-inflammatory activities (12).

CONCLUSIONS

In conclusion, the present study has demonstrated the potency of aqueous extract of Brassica rapa (Br) to ameliorate hyperglycemia, hyperlipidemia and insulin resistance in diabetic rats. Brassica extract suppressed oxidative and inflammation stress in vivo in plasma and aorta and heart tissue in rats fed a high fat-sucrose diet. These results suggest that aqueous extract of Brassica rapa (Br) may have an important implications for the prevention and early treatment of T2DM.

REFERENCES:


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MECHANISTIC MODELS IN THE ASSESSMENT OF DROTAVERINE HYDROCHLORIDE SOLUBILITY AFTER TABLET DISINTEGRATION IN THE PRESENCE OF SELECTED EXCIPIENTS IN PHARMACOPOEIAL ACCEPTOR FLUID

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2Department of Pharmacy, Chair of Applied Pharmacy, Medical University, Muszyńskiego 1, 90-151 Łódź, Poland
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Abstract: Research studies have been carried out on the possibility of producing a solid dosage form with drotaverine hydrochloride by direct compression technique with the use of individual excipients such as: Vivapur, Prosolv 90 HD, Emdex, Emcompress, lactose and Pearlitol 200 SD. Granulometric parameters of tablet mass, model dosage forms as well as a reference form were analyzed. The determined pharmaceutical availability in the form of a Q% coefficient was in the time function (t, min) an inspiration for estimating the rate of dissolution of the therapeutic agent in the presence of an excipient and for assessing the impact of the grain structure and surface in the environment of 0.1 M of HCl on the process of its sorption. Kinetic equations of “0” and “1” order and Higuchi, Korsmeyer-Peppas and Hixson-Crowell models, based on the thesis that the process of dissolution (release) is consistent with Fick’s law, were used to describe the process of dissolution of drotaverine hydrochloride in 0.1 M HCl. It results from the analysis of approximation equations, both in the kinetic and mechanistic model (Higuchi, Korsmeyer-Peppas, Hixson-Crowell models), that symmetrically to the dissolution process there comes to adsorption of the therapeutic agent by granulometric grains which points to its deficit in the acceptor fluid (Freundlich adsorption isotherm). Formulation with Prosolv 90HD is characterized by a profile of the rate of dissolution of drotaverine hydrochloride that is compatible with a reference form. Granulometric combination of lactose with Vivapur 101 is also worthy of note from the application point of view.

Keywords: drotaverine hydrochloride, excipients, tablet, pharmaceutical availability, mathematical models

Compatibility of drotaverine hydrochloride anion with human gastric juice makes the solubility of hydrotropic adduct in vivo a condition which should translate into optimal biopharmaceutical parameters of the type t_{max}, c_{max} and the AVC area (1-3).

The ability to generate amorphous structures – in the process of formulation of a pharmaceutical agent solid form – which significantly alter the rate of therapeutic agent dissolution and balance the adsorption isotherm described by the Freundlich equation (x/m = K · c) is the morphological feature of the crystallographic system of therapeutic agent hydrochlorides (hydrotropic adduct) (4-7).

The amorphous form of the therapeutic agent – in the solid state, has a high value of the internal enthalpy, thus devoid of its own crystal system, and thermodynamically, it will tend to crystallize in the process of compression (8-14).

Applicatively amorphous systems are characterized by enhanced solubility, which favors significant improvement of in vitro and in vivo bioavailability.

Maintaining thermodynamic stability of the amorphous system in the process of production is possible by experimental selection of excipients in appropriate proportion or their individual share in explicit granulometric form (15-21).

* Corresponding author: e-mail: grzegorz.dolega@sanofi.com
The aim of the research studies of application nature was to produce a model dosage form with drotaverine hydrochloride by direct compression technique in different compression conditions with individual excipient and to evaluate their impact on the rate of dissolution process and the rate of mass transfer through the phase boundary.

The model tablet masses (granulates) were subjected to morphological evaluation and there were made tablets of the hardness in the range of 35-45 N (22, 23).

The determination of drotaverine hydrochloride pharmaceutical availability in 0.1 M HCl was performed and the obtained results were the basis for the use of model systems of mathematical equations for reliable assessment of the complex process of the effect of the excipients on the dissolution rate of the therapeutic agent (24-28).

The obtained results enable optimal design and production of therapeutically effective solid oral dosage form i.e., hydrotropic structure of a therapeutic agent of high solubility in model pharmacopoeial acceptor fluids (29, 30).

**EXPERIMENTAL**

**Reagents**

Therapeutic agent: drotaverine hydrochloride: 6,7-diethoxy-1-[(3,4-diethoxyphenyl)methylene]-1,2,3,4-tetrahydroisoquinoline hydrochloride; C_{24}H_{32}ClNO_{4}

Excipients: Microcrystalline cellulose, Ph. Eur., NP., JP: Vivapur 101 (ϕ = 50-65 μm, d = 0.26-0.31 g/cm³), Vivapur 102 (ϕ = 90-100 μm, d = 0.28-0.33 g/cm³), Vivapur 200 (ϕ=190-250 μm, d = 0.31-0.33 g/cm³), Vivapur 12 (ϕ = 160-180 μm, d = 0.30-0.36 g/cm³); C_{6n}H_{10n+20}N_{n+1}, J. Rettenmaier and Son (Germany) (31). Prosolv HD 90, (silificed microcrystalline cellulose with 2% silicon oxide, ϕ = 90 μm, d = 0.42 g/cm³), J. Rettenmaier and Son (31). Lactose, C_{12}H_{22}O_{11}, Borcula Domo-Holland, FP VI, Eur. Pharm. VI.

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**Table 1. Prescription composition of model tablets with drotaverine hydrochloride produced by direct compression method.**

<table>
<thead>
<tr>
<th>Formulation No.</th>
<th>Excipients</th>
<th>Drotaverine hydrochloride</th>
<th>Lactose</th>
<th>Vivapur 101</th>
<th>Vivapur 102</th>
<th>Vivapur 12</th>
<th>Prosolv 90 HD</th>
<th>Emcompress</th>
<th>Pearitol 200 SD</th>
<th>Corn starch</th>
<th>Povidone K-25</th>
<th>Talc</th>
<th>Magnesium stearate</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>○</td>
<td>●●●</td>
<td>●●●●●</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. 2</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>+</td>
<td>+</td>
<td>●●●</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. 3</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>+</td>
<td>+</td>
<td>●●●</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. 4</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
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</tr>
<tr>
<td>No. 5</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>+</td>
<td>+</td>
<td>●●●</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. 6</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. 7</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>+</td>
<td>+</td>
<td>●●●</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. 8</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No. 9</td>
<td>40 mg</td>
<td></td>
<td>+</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>+</td>
<td>+</td>
<td>●●●</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Granulometric parameters of tablet mass prepared for direct compression.

<table>
<thead>
<tr>
<th>Granulometric parameters</th>
<th>Reference formulation</th>
<th>Formulation No. 1</th>
<th>Formulation No. 2</th>
<th>Formulation No. 3</th>
<th>Formulation No. 4</th>
<th>Formulation No. 5</th>
<th>Formulation No. 6</th>
<th>Formulation No. 7</th>
<th>Formulation No. 8</th>
<th>Formulation No. 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angle of repose [°]</td>
<td>42.1</td>
<td>32.8</td>
<td>38.1</td>
<td>30.7</td>
<td>36.2</td>
<td>44.0</td>
<td>40.4</td>
<td>38.6</td>
<td>39.8</td>
<td>39.4</td>
</tr>
<tr>
<td>Flowability s/100g</td>
<td>56.5</td>
<td>96.4</td>
<td>83.2</td>
<td>39.5</td>
<td>48.9</td>
<td>21.8</td>
<td>22.3</td>
<td>25.2</td>
<td>25.8</td>
<td>26.6</td>
</tr>
<tr>
<td>Bulk weight [g/mL]</td>
<td>0.516</td>
<td>0.472</td>
<td>0.374</td>
<td>0.385</td>
<td>0.416</td>
<td>0.428</td>
<td>0.510</td>
<td>0.632</td>
<td>0.836</td>
<td>0.492</td>
</tr>
<tr>
<td>Weight after tapping [g/mL]</td>
<td>0.694</td>
<td>0.659</td>
<td>0.502</td>
<td>0.552</td>
<td>0.537</td>
<td>0.567</td>
<td>0.595</td>
<td>0.766</td>
<td>0.926</td>
<td>0.598</td>
</tr>
<tr>
<td>Carr index</td>
<td>25.65</td>
<td>28.38</td>
<td>25.50</td>
<td>26.25</td>
<td>27.40</td>
<td>24.51</td>
<td>14.29</td>
<td>17.49</td>
<td>9.72</td>
<td>17.73</td>
</tr>
<tr>
<td>Mass moisture [%]</td>
<td>5.18</td>
<td>3.29</td>
<td>4.34</td>
<td>4.23</td>
<td>4.50</td>
<td>4.76</td>
<td>4.62</td>
<td>7.50</td>
<td>5.67</td>
<td>2.04</td>
</tr>
<tr>
<td>d = 2r [mm]</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>mt [mg]</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
</tr>
</tbody>
</table>

Table 3. Morphological parameters of tablets with drotaverine hydrochloride produced by direct compression method.

<table>
<thead>
<tr>
<th>Tablet morphological parameters</th>
<th>Reference formulation</th>
<th>Formulation No. 1</th>
<th>Formulation No. 2</th>
<th>Formulation No. 3</th>
<th>Formulation No. 4</th>
<th>Formulation No. 5</th>
<th>Formulation No. 6</th>
<th>Formulation No. 7</th>
<th>Formulation No. 8</th>
<th>Formulation No. 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied compression - limit 35-45N</td>
<td>35.7 [-2]</td>
<td>41.2 [-4]</td>
<td>43.7 [-12]</td>
<td>44.8 [-9]</td>
<td>43.5 [-7]</td>
<td>42.6 [-7]</td>
<td>42.7 [-8]</td>
<td>44.7 [-3]</td>
<td>43.4 [+8]</td>
<td>42.3 [-3]</td>
</tr>
<tr>
<td>Compression and hardness [N]</td>
<td>34.8</td>
<td>60.7</td>
<td>106.3</td>
<td>87.6</td>
<td>82.8</td>
<td>83.2</td>
<td>83.6</td>
<td>59.7</td>
<td>o</td>
<td>59.9</td>
</tr>
<tr>
<td>Friability (%)</td>
<td>0.35</td>
<td>0.07</td>
<td>0.07</td>
<td>0.00</td>
<td>0.07</td>
<td>0.13</td>
<td>0.00</td>
<td>0.21</td>
<td>0.28</td>
<td>0.07</td>
</tr>
<tr>
<td>Disintegration time [min]</td>
<td>11.33</td>
<td>25.19</td>
<td>12.02</td>
<td>21.35</td>
<td>10.31</td>
<td>6.56</td>
<td>12.10</td>
<td>18.01</td>
<td>&gt;120</td>
<td>23.15</td>
</tr>
</tbody>
</table>
Table 4. Pharmaceutical availability - release coefficient Q of drotaverine hydrochloride from model dosage form.

<table>
<thead>
<tr>
<th>t, min √</th>
<th>Reference formulation</th>
<th>Formulation No. 1</th>
<th>Formulation No. 2</th>
<th>Formulation No. 3</th>
<th>Formulation No. 4</th>
<th>Formulation No. 5</th>
<th>Formulation No. 6</th>
<th>Formulation No. 7</th>
<th>Formulation No. 8</th>
<th>Formulation No. 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/2.236</td>
<td>35.65</td>
<td>35.06</td>
<td>28.94</td>
<td>25.68</td>
<td>21.43</td>
<td>22.65</td>
<td>37.92</td>
<td>23.30</td>
<td>15.46</td>
<td>24.72</td>
</tr>
<tr>
<td>10/3.162</td>
<td>64.46</td>
<td>61.35</td>
<td>49.55</td>
<td>45.45</td>
<td>36.41</td>
<td>38.43</td>
<td>67.12</td>
<td>40.87</td>
<td>29.34</td>
<td>47.24</td>
</tr>
<tr>
<td>15/3.872</td>
<td>85.54</td>
<td>80.00</td>
<td>64.57</td>
<td>61.22</td>
<td>47.98</td>
<td>49.78</td>
<td>85.93</td>
<td>55.36</td>
<td>41.46</td>
<td>62.89</td>
</tr>
<tr>
<td>20/4.472</td>
<td>94.31</td>
<td>93.62</td>
<td>77.65</td>
<td>73.29</td>
<td>55.91</td>
<td>59.15</td>
<td>96.77</td>
<td>67.52</td>
<td>53.49</td>
<td>74.08</td>
</tr>
<tr>
<td>25/5.000</td>
<td>99.12</td>
<td>101.94</td>
<td>87.87</td>
<td>84.87</td>
<td>63.40</td>
<td>65.94</td>
<td>101.92</td>
<td>77.91</td>
<td>64.25</td>
<td>84.47</td>
</tr>
<tr>
<td>30/5.477</td>
<td>103.66</td>
<td>106.57</td>
<td>93.99</td>
<td>92.58</td>
<td>70.32</td>
<td>72.12</td>
<td>103.11</td>
<td>85.36</td>
<td>84.47</td>
<td>88.38</td>
</tr>
</tbody>
</table>

* Numerical values of the release coefficient Q are the mean of 3-5 measurements.
Mechanistic models in the assessment of drotaverine hydrochloride...  

– determination of disintegration time (– disintegration test apparatus Erweka).

Prescription composition of model formulations and the determined and calculated morphological parameters are demonstrated in Tables 1-3.

Profiles of drotaverine hydrochloride pharmaceutical availability from model tablets in the environment of pharmacopoeial acceptor fluids

The determination of the rate of dissolution of drotaverine hydrochloride from model tablets in the...

1. Phase of tablet disintegration

![Figure 1. Physicochemical processes observed during tablet disintegration in the presence of granulometrically defined excipient grain](image)

2. Mixed phase after the process of tablet disintegration

![Figure 1. Physicochemical processes observed during tablet disintegration in the presence of granulometrically defined excipient grain](image)
Table 5. Correlation equations describing the kinetics of drotaverine hydrochloride solubility in 0.1 M HCl in the presence of an excipient, p = 0.05

<table>
<thead>
<tr>
<th>Medium formulation</th>
<th>Function</th>
<th>Equation</th>
<th>Correlation coefficient ( r^2 )</th>
<th>Equation slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference formulation 0.1 M HCl</td>
<td>( Q = f(t) )</td>
<td>( \log y = a + b\cdot 1/x ) ( y = a + b\cdot \log x )</td>
<td>0.9983</td>
<td>2.1098</td>
</tr>
<tr>
<td></td>
<td>( Q = f(\sqrt{t}) )</td>
<td>( y = a + b\cdot 1/x ) ( \log y = a + b\cdot \log x )</td>
<td>0.9964</td>
<td>151.7962</td>
</tr>
<tr>
<td></td>
<td>( \sqrt{100 - Q} = f(t) )</td>
<td>( y = a + b\cdot \log x )</td>
<td>0.9591</td>
<td>6.8157</td>
</tr>
<tr>
<td>Formulation No. 1 0.1 M HCl x</td>
<td>( Q = f(t) )</td>
<td>( y = a + b\cdot \log x )</td>
<td>0.9921</td>
<td>-24.6948</td>
</tr>
<tr>
<td></td>
<td>( Q = f(\sqrt{t}) )</td>
<td>( y = a + b\cdot 1/x ) ( \log y = a + b\cdot \log x )</td>
<td>0.9992</td>
<td>2.3787</td>
</tr>
<tr>
<td></td>
<td>( \sqrt{100 - Q} = f(t) )</td>
<td>( y = a + b\cdot \log x ) ( y = a + b\cdot y )</td>
<td>0.9451</td>
<td>6.5638</td>
</tr>
<tr>
<td>Formulation No. 2 0.1 M HCl</td>
<td>( Q = f(t) )</td>
<td>( y = a + b\cdot \log x )</td>
<td>0.9960</td>
<td>-33.1367</td>
</tr>
<tr>
<td></td>
<td>( Q = f(\sqrt{t}) )</td>
<td>( y = a + b\cdot 1/x ) ( \log y = a + b\cdot \log x )</td>
<td>0.9961</td>
<td>2.3342</td>
</tr>
<tr>
<td></td>
<td>( \sqrt{100 - Q} = f(t) )</td>
<td>( y = a + b\cdot \log x ) ( y = a + b\cdot b )</td>
<td>0.9451</td>
<td>6.5638</td>
</tr>
<tr>
<td>Formulation No. 3 0.1 M HCl</td>
<td>( Q = f(t) )</td>
<td>( \log y = a + b\cdot \log x ) ( y = a + b\cdot x )</td>
<td>0.9938</td>
<td>0.9233</td>
</tr>
<tr>
<td></td>
<td>( Q = f(\sqrt{t}) )</td>
<td>( y = a + b\cdot \log x )</td>
<td>0.9994</td>
<td>-20.4866</td>
</tr>
<tr>
<td></td>
<td>( \sqrt{100 - Q} = f(t) )</td>
<td>( y = a + b\cdot \log x ) ( y = a + b\cdot x )</td>
<td>0.9451</td>
<td>6.5638</td>
</tr>
<tr>
<td>Formulation No. 4 0.1 M HCl</td>
<td>( Q = f(t) )</td>
<td>( \log y = a + b\cdot \log x ) ( y = a + b\cdot x )</td>
<td>0.9967</td>
<td>-23.3011</td>
</tr>
<tr>
<td></td>
<td>( Q = f(\sqrt{t}) )</td>
<td>( y = a + b\cdot \log x )</td>
<td>0.9959</td>
<td>0.9234</td>
</tr>
<tr>
<td></td>
<td>( \sqrt{100 - Q} = f(t) )</td>
<td>( y = a + b\cdot \log x ) ( y = a + b\cdot \log y ) ( \log y = a + b\cdot \log x )</td>
<td>0.9992</td>
<td>-11.3131</td>
</tr>
<tr>
<td>Formulation No. 5 0.1 M HCl</td>
<td>( Q = f(t) )</td>
<td>( \log y = a + b\cdot \log x ) ( y = a + b\cdot x ) ( \log y = a + b\cdot x )</td>
<td>0.9997</td>
<td>-24.1156</td>
</tr>
<tr>
<td></td>
<td>( Q = f(\sqrt{t}) )</td>
<td>( y = a + b\cdot \log x )</td>
<td>0.9996</td>
<td>-20.7998</td>
</tr>
<tr>
<td></td>
<td>( \sqrt{100 - Q} = f(t) )</td>
<td>( y = a + b\cdot \log x ) ( y = a + b\cdot x ) ( \log y = a + b\cdot \log y )</td>
<td>0.9981</td>
<td>4.6942</td>
</tr>
<tr>
<td>Formulation No. 6 0.1 M HCl</td>
<td>( Q = f(t) )</td>
<td>( \log y = a + b\cdot \log x ) ( y = a + b\cdot x ) ( y = a + b\cdot \log y )</td>
<td>0.9994</td>
<td>-24.1156</td>
</tr>
<tr>
<td></td>
<td>( Q = f(\sqrt{t}) )</td>
<td>( y = a + b\cdot \log x )</td>
<td>0.9996</td>
<td>-20.7998</td>
</tr>
<tr>
<td></td>
<td>( \sqrt{100 - Q} = f(t) )</td>
<td>( y = a + b\cdot \log x ) ( y = a + b\cdot \log x ) ( \log y = a + b\cdot \log x ) ( y = a + b\cdot \log y )</td>
<td>0.9992</td>
<td>4.6942</td>
</tr>
</tbody>
</table>
Table 5. Cont.

<table>
<thead>
<tr>
<th>Medium formulation</th>
<th>Function</th>
<th>Equation</th>
<th>Correlation coefficient</th>
<th>Equation slope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation No. 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M HCl</td>
<td>$Q = F(t)$</td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9979</td>
<td>0.8698</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot x$</td>
<td>0.9922</td>
<td>-36.4508</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9901</td>
<td>15.0286</td>
</tr>
<tr>
<td></td>
<td>$Q = f(\sqrt{t})$</td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9995</td>
<td>-20.1652</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9979</td>
<td>0.8698</td>
</tr>
<tr>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9974</td>
<td>2.3091</td>
<td>-2.1381</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9711</td>
<td>6.6663</td>
</tr>
<tr>
<td></td>
<td>$Q = f(\sqrt{t})$</td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9452</td>
<td>1.1163</td>
</tr>
<tr>
<td>Formulation No. 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M HCl</td>
<td>$Q = F(t)$</td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9982</td>
<td>5.4006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9969</td>
<td>0.5843</td>
</tr>
<tr>
<td></td>
<td>$Q = f(\sqrt{t})$</td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9932</td>
<td>0.7406</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9982</td>
<td>0.5446</td>
</tr>
<tr>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9868</td>
<td>2.3430</td>
<td>2.6542</td>
</tr>
<tr>
<td></td>
<td>$\sqrt{100 - Q} = f(t)$</td>
<td>$y = a + b \cdot x$</td>
<td>0.9998</td>
<td>4.6199</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot x$</td>
<td>0.9956</td>
<td>0.6853</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot x$</td>
<td>0.9851</td>
<td>0.1890</td>
</tr>
<tr>
<td>Formulation No. 9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M HCl</td>
<td>$Q = F(t)$</td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9983</td>
<td>-32.2177</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9942</td>
<td>2.0384</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9893</td>
<td>0.9252</td>
</tr>
<tr>
<td></td>
<td>$Q = f(\sqrt{t})$</td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9996</td>
<td>2.3434</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9983</td>
<td>-35.2103</td>
</tr>
<tr>
<td></td>
<td>$y = a + b \cdot \log x$</td>
<td>0.9934</td>
<td>17.2464</td>
<td>20.0362</td>
</tr>
<tr>
<td></td>
<td>$\sqrt{100 - Q} = f(t)$</td>
<td>$y = a + b \cdot x$</td>
<td>0.9682</td>
<td>4.8585</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot x$</td>
<td>0.9430</td>
<td>0.7085</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$y = a + b \cdot x$</td>
<td>0.9118</td>
<td>0.1779</td>
</tr>
</tbody>
</table>

The presence of excipients was performed according to the standard pharmacopeia by a basket method (the volume of acceptor fluid 0.1 M HCl – 1000 cm³, 50 rot/min) using a system for testing the release off line with the collector of Varian fraction connected with the apparatus of high performance chromatography Agilent 1100 series.

The numerical value of the absorbance of the standard solution and the tested solution was read at a wavelength $\lambda = 353$ nm.

The amount of drotaverine hydrochloride (x, Q%) released from the tablet and dissolved in the model acceptor fluid was calculated from the dependence:

$$x(Q\%) = \frac{A_s \cdot m_s \cdot P_s \cdot 2.5}{A_e \cdot 40}$$

where: $A_s$ – test sample absorbance, $m_s$ – weight of the standard (mg), $A_e$ – standard sample absorbance, $P_s$ – content of drotaverine hydrochloride in the reference form 40 – declared content of the therapeutic agent in a single tablet, 2.5 – dilution coefficient.

The calculated release coefficient Q(%) was the base for the application of mathematical models, which in a reliable way enable to estimate the dynamics of the process of dissolution of drotaverine hydrochloride in the presence of excipients in the pharmacopeial acceptor fluid.

The obtained results are demonstrated in Table 4.

**Kinetic models of drotaverine hydrochloride pharmaceutical availability**

The process of pharmaceutical availability (Q%) of model formulations with drotaverine hydrochloride (hydrotropic adduct of the therapeutic agent) should take place in acceptor fluid symmetrically to a progressing disintegration and developed in the time (t, min) surface of the tablet mass.

Thus, mechanistic models based on Fick’s equation as well as kinetic models based on physical laws were applied for the complex process of dissolution of drotaverine hydrochloride in the presence of excipients of different granulometric parameters.

Taking the above into consideration, the following model equations were applied to describe the rate of the therapeutic agent dissolution:

$$Q(\%) = f(t,\text{min})$$

$$Q(\%) = a + k \cdot t$$

$$\log Q(\%) = f(t,\text{min})$$

$$\log Q(\%) = a + k \cdot t$$

0 and 1-order kinetics equations.
(3) \( Q(\%) = f(\sqrt{t}) \); Higuchi model \( Q(\%) = k \cdot t^{1/2} \)  
which is the base for Fick’s diffusion model,

(4) \( \log Q(\%) = f(\log t) \); Korsmeyer-Peppas model  
\( Q(\%) = k \cdot t^n \), which in the logarithmic system has the form \( \log Q(\%) = \log K + n \log t \) (applicatively the used form is \( Q(\%) = a = n \log t \); where \( a = \log K \)),

(5) \( Q_{01/3}(\%) = Q_t(\%) = K_{H} t \); Hixson-Crowell,

which in application has the form \( Q_t(\%) = f(t, \text{min}) \).

It is assumed in kinetic models of 0 and 1-order that the rate of therapeutic agent release is independent of the function of time \( (t, \text{min}) \). However, in the Korsmeyer-Peppas model and in Higuchi model, which are based on the thesis that the release process is compatible with Fick’s law, its rate varies with time.

---

Figure 2. The course of correlation between pharmaceutical availability – \( Q\% \) and disintegration time of a model tablet in time function – \( t, \text{min.} \); \( Q\% = f(t, \text{min.}) \).

Figure 3. The course of correlation between pharmaceutical availability – \( Q\% \), and \( \sqrt{t} \) in Higuichi model: \( Q\% = f(\sqrt{t}, \text{min.}) \).
RESULTS AND DISCUSSION

It results from prescription compositions of the model dosage form, demonstrated in Table 1, that using the optimal content of talc and magnesium stearate as a lubricant in the process of effective compression, there was used for their production only one excipient with defined granulometric parameters (formulation No. 2-9). The reference and No. 1 (32) formulations were manufactured with increased share of excipients (Table 1) which was reflected in the parameter assessing the tablet mass flow (flowability) and tapped bulk density (Table 2).

An angle of repose of tablet mass for model formulations remains an interesting application parameter of applicative significance in the process of tablet stamping (formulation No. 2-9) in relation to the reference formulation.

It represents, with symmetrical filling of the niche and lack of the tendency to tablet mass bridging, the condition for statistical distribution of the therapeutic agent in the produced tablet. This is confirmed by the determined morphological parameters of the produced tablets which at comparable compression are characterized by their proper hardness and – with maintained grain granulometric size and the ability to water sorption – the effective disintegration time is its emanation (Table 3).

Comparative studies were conducted in the environment of model pharmacopoeial acceptor fluid (0.1 M HCl) compatible with the hydrotropic form of drotaverine hydrochloride in the dissolution rate of the therapeutic agent in the presence of excipients. The calculated pharmaceutical availability coefficients – Q(%) in the function of exposure time are shown in Table 4. The time range of the carried out research resulted from the electrical disintegration time of this form of tablets (~ 20 min) provided by pharmacopoeial standard (33).

Demonstrated in Table 4 results of pharmaceutical availability in the process of drotaverine hydrochloride dissolution in the presence of excipients in mathematical models (kinetic solubility of 0 and 1-order) and Higuchi and Korsmeyer-Peppas models associated with Fick’s law (34) as well as in Hixson-Crowell model, which takes into account the topological dimension of the therapeutic agent hydrotropic molecule.

Analyzing the processes related to the disintegration of the tablet in the model acceptor fluid (0.1 M HCl, 200 m Osm/dm³), symmetrically ongoing processes associated with drotaverine hydrochloride molecule dissolution (hydration-solvation) should be taken into account, as well as their physical adsorption on the surface of homogeneous adsorbent (excipient) combined with filling of the granulometric grain space with a solution the structure of which is not biodegradable during GI tract transit (35, 36). With the significant concentration gradient between the solution in the structure of the granulometric grain and the external solution (acceptor fluid), the equilibrium in the concentrations will be reached during the mixing process. In this situation, the equation in the mathematical model of Korsmeyer-Peppas ~Q(%) = k ∑ tⁿ represents a significantly compatible reference to the empirical Freundlich equation – y (x/m) = k ∑ cⁿ, which describes the process of physical adsorption of the therapeutic agent on the surface of the excipient (solid adsorbent), Figure 1.

The demonstrated in Table 4 calculated coefficients of release Q(%) were the basis for tracing the dependence Q(%) = f (t, min) (Fig. 2) and for describing their course at a coefficient of probability p = 0.05 with correlation equations which are presented in Table 5.

It results from the analysis of disintegration of model tablets and from the dissolution rate of drotaverine hydrochloride in 0.1 M HCl (Table 4, Fig. 2) that in the kinetic model – Q(%) = f (t, min) their courses formed three independent sets in relation to the reference formulation (RF).

Formulation No.1 (lactose + Vivapur 10) and No.6 (Prosolv 90HD) were found in the first set, symmetrical in relation to the reference formulation. Surprisingly, formulations: No. 2 (Vivapur 101), No. 3 (Vivapur 102), No. 7 (EMDEX) and No. 9 (Perlitor 200 SD) were in the second set, whereas in the third set formulations: No. 4 (Vivapur 200), No. 5 (Vivapur 12) and No. 8 (Emcompress).

It results from Table 5 approximation equations of high correlation coefficient r² ≥ 0.990 that in the kinetic model the equations: y = a + b · log x and log y = a + b · 1/x describe the model formulations compatible with the reference formulation (RF).

However, for other sets, the profile of dependence of which – Q (%) = f (t, min) is similar to Freundlich adsorption isotherm (y = k · c/n) the dominating correlation equation is – log y = a + b · log x supplemented individually for the formulations with the equations of the type: y = a + b · x, y = a + b · log x and log y = a + b 1/x.

In Higuchi model also three sets were noted with the same formulation composition of excipients for the course of functional correlation – Q(%) = f (√t), Fig. 3.
The course of the correlation \(Q(\%)\) in the function of \(\sqrt{t}\) for the reference (RF) and model formulations No.1 and No. 6 is described in the order of numerical value of the correlation coefficient by the equations of the type:

\[
y = a + b \cdot 1/x, \quad \log y = a + b \cdot 1/x, \quad \text{and} \quad y = a + b \cdot \log x
\]

However, for the second and third set consisting, according to the course of the function in the order of model formulations: No. 2, No. 3, No. 9, No. 7, No. 5, No. 4 and No. 8, maintaining the above-mentioned notification, i.e., according to the numerical value of “\(r^2\)” coefficient, their course is described by equations of the type: \(\log y = a + b \cdot \log x\) and \(\log y = a + b \cdot 1/x\) with supplementation of equations of the type: \(y = a + b \cdot \log x\) and \(y = a + b \cdot x(!)\) for individual formulations.

Axiologically reassessing the observed phenomena and processes that accompany the mechanism of drotaverine hydrochloride dissolution in 0.1 M HCl in the presence of excipients, it should be emphasized that on its own only Prosolv 90 HD of particle size 90-110 µm and density 0.35-0.50 g/cm³ (17) makes it possible to produce a model tablet morphologically and kinetically compatible with the reference formulation (RF). Formulation No. 1 is also worth noting with lactose and Vivapur 101 of particle size 50 (65) µm and density 0.26-0.31 g/cm³ in its prescription composition (31).

Thus, the individual use of Vivapur: 101, 102, 12 and 200 in the formulation of a model tablet results in pharmacopoeial conditions (0.1 M HCl) in temporary loss of drotaverine hydrochloride content in the acceptor fluid due to the binding of therapeutic agent solution by the granulometric grain structure as well as in physical adsorption of the therapeutic agent. Microcrystalline cellulose (Vivapur 101-200) is not biodegradable in the gastrointestinal tract but still in time function osmolar equilibrium is determined between the solution in the grain structure and molecular solution in the gastrointestinal tract (small intestine) in the process of mass exchange at the phase boundary.

In the Hixson-Crowell model, which takes into account the topological dimension of therapeutic agent hydrotropic particle \(R_o=\sqrt[3]{\sum \eta} \cdot M_{micel}/0.005,\) \(R_{obs}=\sqrt[3]{0.09549 \sum \eta} \cdot M_{micel}\) (34-36), the course of the correlation \(\sqrt[3]{100}Q(\%) = f (t, \text{min})\) is of regressive character (Fig. 4) with maintained division into three sets.

Particular attention should be paid to the course of the correlation \(\sqrt[3]{100}Q(\%) = f (t, \text{min})\) (Fig. 4) in the class of microcrystalline cellulose Vivapur 101, 102, 200, 12 and Provasol 90 HD in the presence of which the complex process of drotaverine hydrochloride dissolution at substantially high values of the correlation coefficient \(r^2 \geq 0.990\) is described by equations of the type: \(y = a - b \cdot x\) and \(y = a - b \cdot \log x\) supplemented by correlations \(1/y = a - b \cdot x\) and \(\log y = a - b \cdot \log x\).

For excipients soluble in 0.1 M HCl such as EMDEX and Pearlitol 200 SD and insoluble –

![Figure 4. The course of correlation $\sqrt[3]{100}Q$ in time function t, min. for the process of pharmaceutical availability of drotaverine hydrochloride in the model](image-url)
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Emcompress, the course of the correlation in Hixson-Crowell model $\sqrt{100 - Q(\%) = f(t, \text{min})}$ at variable numerical value of the correlation coefficient $r^2$ in the range 0.968 - 0.999 is generally described by equations of the type $y = a - b \cdot x$ and $\log y = a - b \cdot x$, which basically describe the course of the correlation $\sqrt{100 - Q(\%) = f(t, \text{min})}$, do not reflect in full the processes associated with the physicochemical and granulometric properties of the excipients.

CONCLUSIONS

1. The obtained results of the research have demonstrated that only SiO$_2$ silicified microcrystalline cellulose Prosolv 90H allows independent production of model dosage form with drotaverine hydrochloride of morphological and kinetic parameters compatible with the reference formulation by direct compression technique.

Applicative combination of lactose with Vivapur 10 is worth noting, which also allows to produce a model dosage form of expected morphological and pharmacokinetic parameters.

2. Analyzing the course of the correlation in the kinetic model $-Q(\%) = f(t, \text{min})$, Higuchi model $-Q(\%) = f(t)$ and in Korsmeyer-Peppas model $-Q(\%) = f(\log t)$ Table 4 – with the whole complexity of the process of drotaverine hydrochloride dissolution in 0.1 M HCl in the presence of excipients such as Vivapur, EMDEX, Emcompress and Pearlitol 200 SD, symmetrical in time process of adsorption of the therapeutic agent solution by granulometric grain (Freundlich adsorption isotherm) should be noted, which is manifested by its deficit in the acceptor fluid. This is fully reflected in approximation equations demonstrated in Table 4. It results from the carried out studies that excipients such as Vivapur, EMDEX, Emcompress and Pearlitol 200SD require in application formulations the use of other excipients (e.g., sugar alcohols) which decrease their ability to sorption.

REFERENCES

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Limited gastric residence time has complicated the oral sustained drug delivery system. As the majority of the drugs are better absorbed from the stomach or the upper part of small intestine, both prevention in complete drug release in the absorption zone and reduction in the efficacy of administered dose can occur due to the rapid gastrointestinal transit (1, 2). For a number of drugs showing limited bioavailability as a result of having narrow absorption window in the upper segment of GIT, hydrodynamically balanced system (HBS); one of the advancements of floating drug delivery approach promises various applications. It helps in prolonging the gastric residence time (GRT) and increasing the quantity of drug that reaches at its site of absorption to a maximum level, thereby improving bioavailability (3).

A unique antidepressant, venlafaxine (1-[2-(dimethylamino)-1-(4-methoxyphenyl)ethyl cyclohexanol hydrochloride) is referred to as a serotonin, norepinephrine-dopamine reuptake inhibitor (4). Venlafaxine acts as an antidepressant by inhibiting the neuronal uptake of serotonin, norepinephrine and to a lesser degree dopamine (5, 6). Venlafaxine has a very short steady state half-life of approximately 5 ± 2 h. So in order to maintain the adequate plasma levels of the drug, two or three times daily administration is required (7). Due to its short half-life, patients are also directed to strictly comply with the medication regimen and not to miss even a single dose, as missing a dose can produce withdrawal symptoms (8). In this situation, such a dosage form is necessary which is capable of releasing the drug in sustained manner that will also simplify the therapeutic regimen by avoiding the requirement to administer the formulation frequently. Fewer incidences of nausea and dizziness have also been reported with the use of such types of formulations (9). The bioavailability of venlafaxine is low ranging from 10-45%. According to BCS, it falls under the class I since it is having high solubility and high permeability. It is also freely soluble in water (572 mg/mL) (10). These properties of venlafaxine together with the requirement of reduction in its frequency of administration make it an ideal candidate for the development of sustained release gastroretentive dosage form.

FORMULATION, DEVELOPMENT AND IN VITRO EVALUATION OF A NOVEL SUSTAINED RELEASE HYDRODYNAMICALLY BALANCED GASTRORETENTIVE DOSAGE FORM FOR VENLAFAXINE HCl

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Abstract: The present study was aimed to formulate, develop and evaluate hydrodynamically balanced system of an antidepressant, venlafaxine hydrochloride as a single unit floating capsule by the application of Box-Behnken statistical design with 3 factors, 3 levels and 15 runs. Formulations were prepared by physical blending of the drug and polymers in varying ratios. The contents of hydroxyl propylmethylcellulose K15M (A), ethylcellulose (B) and cellulose acetate phthalate (C) were used as independent variables whereas the dependent variables considered were the cumulative percentage drug release at 2 h (Y1), 8 h (Y2), 12 h (Y3) and total floating time (Y4). Mathematical model was used to evaluate the main effects and interaction terms quantitatively. Formulation optimization was done by setting the target values for the response variables. The optimized formulation was observed to fulfill the requirements of an optimum controlled release floating dosage form since it better regulated the drug release (Y1; 4.28%, Y2; 70.83% and Y3; 80.92%) and also floated well for more than 16 h. It can be concluded that the hydrodynamically balanced system of venlafaxine hydrochloride can be successfully formulated as an approach to increase the gastric residence time and hence improving its bioavailability.

Keywords: hydrodynamically balanced system, venlafaxine HCl, Box-Behnken statistical design, formulation optimization

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Response surface methodology is extensively used to optimize formulations by the selection of a suitable experimental design because by using it, a deeper understanding of a process or product is obtained as well as robustness of that product can be established (11). Box-Behnken statistical design is an advancement from factorial designs that have been used in response surface modeling and optimization so widely (12, 13).

This study was conducted with an aim to develop floating gastroretentive capsule formulation incorporating 150 mg venlafaxine HCl along with swellable hydrophilic polymer HPMC K15M and release modifiers such as EC and CAP which would release the drug in stomach and upper part of GIT in a controlled manner. Since venlafaxine has better absorption from these regions, gastroretention of the dosage form will improve its oral bioavailability.

MATERIALS AND METHODS

Materials
Venlafaxine HCl was received as a gift sample from M/s Global Pharmaceuticals (Pvt.) Ltd., plot 22-23, Industrial triangle Kahuta road, Islamabad, Pakistan. HPMC K4M, HPMCK15M (hydroxyl propylmethylcellulose), EC (ethylcellulose), CAP (cellulose acetate phthalate), acetone extra pure (99%) and HCl (37% pure) were obtained from Sigma-Aldrich Laborchemikalien GmbH. D-30926 Seeize. Empty hard gelatin capsule shells (size # 00) were obtained from Qaiser Scientific store, Lahore, Pakistan.

Drugs-excipients interaction study and identification

Fourier transform infrared spectroscopy (FTIR)
This study was done by adopting the method described previously (14). An infrared spectrum of pure drug, each excipient and physical mixture of optimized formulation was recorded using FTIR spectrophotometer (IR Prestige 21, Shimadzu). The scanning range was 4000-500 cm⁻¹ and the IR spectra of samples were obtained using KBr disc method. To detect any chemical interaction, any alteration in the spectrum pattern of drug due to presence of polymers was investigated.

UV spectroscopy (determination of λ_max)
As described by Pawar HA and Dhavale R (14), the UV spectrum of venlafaxine HCl solution (concentration; 3 µg/mL) in 0.1 M HCl (pH 1.2) was recorded in the range of 200-400 nm on double beam UV-visible spectrophotometer (UV 1700 Shimadzu). The spectrum and wavelength of maximum absorption were recorded.

Preparation of standard curve
For this purpose, procedure used by (14) was used with some modifications. The absorbance of venlafaxine HCl solutions (concentration; 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 and 33 µg/mL) was measured at 225 nm against blank i.e., 0.1 M HCl on double beam UV-visible spectrophotometer (UV 1700 Shimadzu). The coefficient of correlation and equation for the straight line were also determined.
**Box-Behnken statistical design**

One of the widely used response surface designs, a Box-Behnken statistical design with 3 factors, 3 levels, and 15 runs with triplicate center points was employed for the formulation of hydrodynamically balanced system of venlafaxine HCl. Formulation design, optimization and other investigations were done using Statgraphics Centurion XVI software from Stat-Point Technologies, USA, version 15.2.0 (64 bit). The independent variables or the factors were the amount of HPMC K15M (A), EC (B) and CAP (C). Levels of these factors were set in the formulation design on the basis of the results of preliminary study and were coded as -1, 0, and +1 as shown in Table 1. The responses selected were the cumulative percentage drug release at 2 h (Y1), 8 h (Y2) and 12 h (Y3) and the total floating time (Y4). Formulation optimization was done by setting targets for these response variables.

**Preparation of capsules**

**Preparation of EC granules**

Firstly, EC granules were prepared by weighing 50 mg of EC and then dissolving it in 1 mL of acetone. Then, this solution was granulated with already weighed drug content. After this, the granules were air dried, passed through ASTM # 25 mesh and finally the granules equivalent to 150 mg of venlafaxine HCl were weighed separately. Similar procedure was adopted for preparing EC granules containing 100 mg and 150 mg of EC.

**Filling of capsules**

By the use of low density floating polymer such as HPMC K15M and release modifying agents such as EC and CAP, single unit floating capsules of venlafaxine HCl were formulated that would achieve a density lower than the gastric fluids after administration and hence would float. The composition of designed 15 formulations has been listed in Table 2. EC granules equivalent to one hundred and fifty milligrams of venlafaxine HCl were weighed separately. Similar procedure was adopted for preparing EC granules containing 100 mg and 150 mg of EC.

**Evaluation of in vitro floating properties**

The in vitro floating properties of HBS capsules were determined visually. The capsules were immersed in 900 mL of 0.1 M HCl (pH 1.2) in USP type II apparatus (Pharma test). The operating speed for the paddle was 50 rpm and the apparatus was maintained at temperature 37 ± 5°C. The time required for the capsules to rise to the surface was taken as floating lag time and the time for which the capsules constantly remained buoyant on the surface of the medium was taken as the total floating time (Y4).

**Drug release kinetics**

The kinetic analysis of in vitro release data of venlafaxine HCl from different HBS capsules was done by fitting it into various mathematical models such as zero order, first order, Higuchi, Hixson and Crowell powder dissolution, and Korsmeyer–Peppas model. By calculating the r² values, the fitness of the data was determined (15). This was done by using excel solver add-in (16).

**Zero-order model**

It describes the systems where the drug release rate is independent of concentration of the dissolved substance (17).

\[ F = k_o \cdot t \] (1)

where, the fraction of drug that has been released in time t is represented by F, and k_o is representing the zero-order release constant and t is time in h.

**First-order model**

It suggests that the drug release rate depends on its concentration (18).
\[ \ln F = K_{1st} t \] (2)

where, the fraction of drug that has been released in time \( t \) is represented by \( F \), and \( K_{1st} \) is representing the first-order release constant and \( t \) is time in h.

**Higuchi model**

It is a square root model used for describing the release of drugs incorporated into semisolid and solid matrices developed by Higuchi et al. (19). It has been shown in the equation given below,

\[ F = K_H \sqrt{t} \] (3)

where, \( F \) represents the fraction of drug released in time \( t \), and \( K_H \) is the Higuchi dissolution constant and \( t \) is time in h.

**Hixson and Crowell powder dissolution model**

This model developed by Hixson and Crowell is represented by the following equation (20),

\[ F = 100 \left( 1 - (1 - K_{HC}t)^3 \right) \] (4)

where, \( F \) represents the fraction of drug released in time \( t \), and \( K_{HC} \) is the dissolution constant and \( t \) is time in h.

**Korsmeyer–Peppas model**

They developed a simple mathematical model, relating exponentially the drug release from a polymeric system to the elapsed time (t) (21).

\[ F = K_{KP} t^n \] (5)

where, \( F \) represents the fraction of drug released in time \( t \), \( K_{KP} \) is the rate (kinetic) constant incorpora-
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RESULTS AND DISCUSSION

Drug-excipients interaction study and identification

Fourier transform infrared spectroscopy (FTIR)

A slight variation was observed in the characteristic bands of FTIR spectrum of venlafaxine HCl after doing pre-formulation studies revealing no chemical interaction. These spectra have been given in Figures 1 and 2.

UV spectroscopy (determination of $\lambda_{\text{max}}$)

The UV spectrum of venlafaxine HCl showed that the wavelength of maximum absorbance was obtained at 225 nm. The UV spectrum has been shown in Figure 3.

Calibration curve of venlafaxine

The calibration curve was found to be linear in the range of 3-33 $\mu$g/mL and straight line equation was obtained having the correlation coefficient value of 0.9935. The calibration data of Venlafaxine has been shown in Table 3 and the calibration curve has been shown in Figure 4.

Formulation development

Initially, HPMC K4M was evaluated for its floating characteristics but satisfactory results were
Figure 5. (A-D). Main effects plot for Y1-Y4, respectively. Where HPMC: hydroxypropylmethyl cellulose, EC: ethyl cellulose and CAP: cellulose acetate phthalate.
not obtained. Then, another grade of HPMC i.e., HPMC K15M was examined and as a result of obtaining desired results, was chosen as a low density floating polymer. After the application of Box-Behnken statistical design, fifteen formulations were prepared and then evaluated for four responses such as Y₁, Y₂, Y₃ and Y₄. \textit{In vitro} drug release and buoyancy study revealed that values of responses for these fifteen formulations varied markedly indicating strong relationship between responses and the factors as shown in Table 4. The range of response Y₁ was from 32.45% in F5 to 43.32% in F11. Response Y₂ ranged from 70.71% in F15 to a maximum of 90.29% in F2. Similarly, another response Y₃ was in the range of 81.18% in F15 to 99.25% in F2 and Y₄ was ranging from 10 h in F2 to 16 h in F1.

\textit{In vitro} drug release

It has been observed that all designed formulations showed high values of Y₁. Such a high release could be due to the probable reason that the gel layer formed by HPMC K15M that is effective in controlling drug release is not generated immediately. As long as the gel barrier is being formed, there is high erosion rate and subsequent high drug release initially (23-25). Then, as the hydration of polymer occurs, the drug release slows down. So, in case of Y₁, amount of HPMC K15M proved to be insignificant (p > 0.05). When ANOVA was performed at 95% confidence interval to estimate the significance of the model, the factors that were significant in controlling drug release at the end of 2 h were the amount of EC (p < 0.05) and the amount of CAP (p < 0.05). It was also observed that Y₁ and both the amount of EC and CAP have an inverse relationship depicting that as the amount of EC and CAP are increased, drug release is decreased and vice versa. For instance, F5 showed the least value of Y₁ (32.45%) as it contained the maximum amount of

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Y₁ (%)</th>
<th>Y₂ (%)</th>
<th>Y₃ (%)</th>
<th>Y₄ (h)</th>
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</thead>
<tbody>
<tr>
<td>F1</td>
<td>32.53</td>
<td>76.35</td>
<td>89.29</td>
<td>16</td>
</tr>
<tr>
<td>F2</td>
<td>36.43</td>
<td>90.29</td>
<td>99.25</td>
<td>10</td>
</tr>
<tr>
<td>F3</td>
<td>39.17</td>
<td>75.27</td>
<td>88.55</td>
<td>14</td>
</tr>
<tr>
<td>F4</td>
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<td>74.94</td>
<td>87.97</td>
<td>10</td>
</tr>
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<td>F5</td>
<td>32.45</td>
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</tr>
<tr>
<td>F6</td>
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<td>83.73</td>
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</tr>
<tr>
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<td>35.35</td>
<td>78.76</td>
<td>89.29</td>
<td>14</td>
</tr>
<tr>
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<td>81.41</td>
<td>93.94</td>
<td>16</td>
</tr>
<tr>
<td>F9</td>
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<td>81.08</td>
<td>89.88</td>
<td>10</td>
</tr>
<tr>
<td>F10</td>
<td>41.91</td>
<td>80.49</td>
<td>92.37</td>
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</tr>
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<td>43.32</td>
<td>83.98</td>
<td>98.17</td>
<td>13</td>
</tr>
<tr>
<td>F12</td>
<td>35.44</td>
<td>80.41</td>
<td>94.19</td>
<td>13</td>
</tr>
<tr>
<td>F13</td>
<td>36.18</td>
<td>80</td>
<td>91.12</td>
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<td>81.58</td>
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</tr>
<tr>
<td>F15</td>
<td>35.19</td>
<td>70.71</td>
<td>81.18</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 3. Calibration data of venlafaxine (n = 4).

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Absorbance</th>
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</thead>
<tbody>
<tr>
<td>3</td>
<td>0.1081 ± 0.00011</td>
</tr>
<tr>
<td>6</td>
<td>0.2043 ± 0.0003</td>
</tr>
<tr>
<td>9</td>
<td>0.3062 ± 0.00023</td>
</tr>
<tr>
<td>12</td>
<td>0.4571 ± 0.0001</td>
</tr>
<tr>
<td>15</td>
<td>0.5350 ± 0.00011</td>
</tr>
<tr>
<td>18</td>
<td>0.6333 ± 0.00021</td>
</tr>
<tr>
<td>21</td>
<td>0.7362 ± 0.00026</td>
</tr>
<tr>
<td>24</td>
<td>0.8025 ± 0.00019</td>
</tr>
<tr>
<td>27</td>
<td>0.9221 ± 0.00011</td>
</tr>
<tr>
<td>30</td>
<td>1.1124 ± 0.00013</td>
</tr>
<tr>
<td>33</td>
<td>1.2050 ± 0.00018</td>
</tr>
</tbody>
</table>

Table 4. Observed responses in designed fifteen HBS formulations.
Figure 6. (A-D). Contour plot for $Y_1$-$Y_4$, respectively. Where HPMC: hydroxypropylmethyl cellulose, EC: ethyl cellulose, CAP: cellulose acetate phthalate and TFT: total floating time. The amounts of HPMC, EC and CAP are in mg. The drug release at 2h, 8 h and 12 h is in %.
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EC 150 mg and CAP 60 mg. F7 showed 35.35% and F12 showed 35.44% of Y1 since both were formulated with EC 100 mg and CAP 40 mg. On the other hand, F11 showed the highest value of Y1 (43.32%) as the amount of EC was 50 mg and CAP 20 mg only.

As the time passed on, the gel layer formed by HPMC K15M became effective and played an important role in controlling drug release at the end of 8 h along with the hydrophobic nature of EC coating as a result of lacking any of the hydrophilic groups (hydroxyl or carboxylic). In case of Y2, the amount of CAP proved to be insignificant (p > 0.05). While the amount of HPMC K15M and the amount of EC was statistically significant (p < 0.05) as determined after performing ANOVA at 95% confidence interval. It was also observed that Y2 and both the amount of HPMC K15M and EC have an inverse relationship depicting that as the amount of HPMC K15M and EC are decreased, drug release is increased and vice versa. For instance, F2 showed the highest value of Y2 (99.25%) as the amount of HPMC K15M and EC were at low level i.e., 200 mg and 50 mg, respectively. F7 showed 89.29%, F13; 91.12% and F12; 90.29% as these were formulated with HPMC K15M 250 mg and EC 100 mg. On the other hand, F2 showed the highest value of Y2 (99.25%) as the amount of HPMC K15M and EC were at low level i.e., 200 mg and 50 mg, respectively.

A majority of the HBS capsules extended the release of venlafaxine HCl for more than 12 h. The complex interaction between diffusion, swelling and erosion mechanisms is known to be involved in the drug release from these hydrophilic matrices. The hydration of HPMC controls all of these mechanisms. It also forms the gel barrier through which the drug diffuses (23). Higher concentration of the polymer causes greater amount of gel to be formed and subsequent increase in the diffusion path length for the drug due to which drug release from the formulation is retarded. Increase in the polymer proportion also cause decrease in the growth of erosion, diffusion and swelling fronts due to the formation of a stronger gel layer and subsequent difficult entry of medium into the matrix (24, 26-28).

After performing ANOVA at 95% confidence interval to estimate the significance of the model, the amount of both HPMC K15M and EC was proved to be significant in controlling the cumulative percentage drug release at 12 h (p < 0.05) and the amount of CAP was insignificant (p > 0.05). It was also observed that Y3 and both the amount of HPMC K15M and EC have an inverse relationship depicting that as the amount of HPMC K15M and EC are decreased, drug release is increased and vice versa. For instance, F2 showed the highest value of Y3 (99.25%) as the amount of HPMC K15M and EC were at low level i.e., 200 mg and 50 mg, respectively. F7 showed 89.29%, F13; 91.12% and F12; 90.29% as these were formulated with HPMC K15M 250 mg and EC 100 mg. On the other hand, F2 showed the highest value of Y2 (99.25%) as the amount of HPMC K15M and EC were at low level i.e., 200 mg and 50 mg, respectively.
94.19% of Y₄, as these were formulated with 250 mg of HPMC K15M and 100 mg of EC. While the least cumulative percentage drug release was shown by F15 (81.18%) since the amount of HPMC K15M and EC was 300 mg and 150 mg, respectively, in that formulation.

**In vitro buoyancy**

A majority of the formulations floated well for more than 12 h. The increased gel strength of the polymeric combination matrices might be the probable reason. The mechanism responsible to make these capsules buoyant could be the rapid hydration and swelling of polymeric matrices resulting in the formation of a floating mass (29). The factors that were found to play significant role in governing Y₄ after performing ANOVA at 95% confidence interval, were A (p < 0.05) and AC (p < 0.05). It was also observed that the matrix integrity of these HBS capsules was satisfactory. Main effects plots and contour plots for different formulation factors have been shown in the Figure 5 (A-D) and Figure 6 (A-D), respectively.

**Drug release kinetics**

Table 5 lists various kinetic models computed for all the HBS gastroretentive formulations. Venlafaxine release data were evaluated by zero-order, first-order, and Higuchi and Hixson-Crowell models. Some of the formulations followed first-order release patterns because the plots of the percent cumulative drug release versus the square root of time were found to be linear. Hence, the mechanism of drug release from these formulations was found to be diffusion-controlled such as F1, F9 and F13. The rest of the formulations followed Higuchi model since the regression coefficient (r²) values are ranging from 0.9509 to 0.9989, suggesting that the release of a solid drug from a hydrophilic matrix involved the simultaneous penetration of the sur-

![Figure 7. Kinetic evaluation of the optimized formulation: Korsmeyer-Peppas plot](image-url)

Table 6. Multiple response optimization.

<table>
<thead>
<tr>
<th>Response</th>
<th>(Range observed in F1 - F15)</th>
<th>Target value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Cumulative release at the end of,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) 2 h</td>
<td>Low 32.45</td>
<td>43.32 minimize</td>
</tr>
<tr>
<td>(ii) 8 h</td>
<td>70.71</td>
<td>90.29 minimize</td>
</tr>
<tr>
<td>(iii) 12 h</td>
<td>81.18</td>
<td>99.25 minimize</td>
</tr>
<tr>
<td>Total floating duration (h)</td>
<td>10</td>
<td>16 maximize</td>
</tr>
</tbody>
</table>

rounding liquid, dissolution of the drug, and leaching out of the drug through interstitial channels or pores. This was further augmented when the release data were subjected to Korsmeyer-Peppas equation which is used to describe the anomalous release behavior from the matrix. In the present study, the values of \( n \), calculated per the algorithm proposed by Peppas and Sahlin (31), ranged between 0.4753–0.6599. The values of the kinetic constant (\( K_1 \)), which is a direct function of matrix solubility, were found to increase with increases in the amount of either polymer (32, 21). It should be noted that \( K_1 \) has much lower values when compared to \( K_0 \), clearly indicating that the release of venlafaxine was primarily controlled by non-Fickian diffusion depicting that swelling and erosion of the HBS gastroretentive capsules go on to increase in association with an increase in the content of any of the polymers.

Data analysis

Mathematical relationships for the measured responses and the independent variables or factors were generated with the help of software Statgraphics Centurion XVI and are shown in Equations 6-9. These equations represent the quantitative effect of variables (A, B, C) and their interactions on the response. Coefficient with more than one factor term and those with higher order terms represent interaction terms and quadratic relationship, respectively. A positive sign represents synergistic effect, while a negative sign indicates antagonistic effect or an inverse relationship between the factors and response (30). Correlation coefficient (\( r^2 \)) for the equations indicates the percentage variability in model fitting for that particular variable. The adjusted \( r^2 \) value is more suitable for comparing models with different number of independent variables which can be obtained by including only statistically significant (p < 0.05) coefficients in the equation. The adjusted \( r^2 \) value for responses \( Y_1, Y_2, Y_3 \) and \( Y_4 \) were found to be 90.78%, 93.64%, 85.02% and 86.02%, respectively, which indicate good fit.

Formulation optimization and evaluation

For formulation optimization, target values for the responses (\( Y_1-Y_4 \)) were set on the basis of \textit{in vitro} drug release and buoyancy study of multiple trial formulations run in the laboratory (Table 6). Composition of the optimized formulation given by the software using this technique known as “multiple response optimization” is shown in Table 7. Optimized formulation was prepared by the method as described before. Then, it was evaluated for \( Y_1, Y_2, Y_3 \) and \( Y_4 \) by the method as described before. Furthermore, the drug release data were analyzed kinetically by fitting into various mathematical models as described above. The results of all these parameters have been shown in Tables 8, 9 and Figure 7. The desirability was maximized by the use of optimized formulation over the indicated region to 0.916206 as shown in Figure 8.

It was also observed that the optimized formulation fulfilled the requirements of an optimum controlled release dosage form since it better regulated the drug release at the end of 2 h (34.28%), 8 h (70.83%) and 12 h (80.92%). The validity of the developed model is indicated by the point that the release profile predicted by the software was found to be very close to that observed experimentally (Table 8). The release profile of the optimized for-

<table>
<thead>
<tr>
<th>Composition</th>
<th>Amount per capsule (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venlafaxine HCl</td>
<td>150</td>
</tr>
<tr>
<td>Amount of HPMC K15M (A)</td>
<td>299.999</td>
</tr>
<tr>
<td>Amount of EC (B)</td>
<td>149.999</td>
</tr>
<tr>
<td>Amount of CAP (C)</td>
<td>59.999</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Response variables</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Y_1 (%) )</td>
<td>33.2004</td>
<td>34.28</td>
</tr>
<tr>
<td>( Y_2 (%) )</td>
<td>71.5634</td>
<td>70.83</td>
</tr>
<tr>
<td>( Y_3 (%) )</td>
<td>81.5411</td>
<td>80.92</td>
</tr>
<tr>
<td>( Y_4 (h) )</td>
<td>16.0417</td>
<td>&gt;16</td>
</tr>
</tbody>
</table>

Table 7. Composition of the optimized formulation.

Table 8. Response variables of the optimized formulation (observed and predicted).
mulation could be best expressed by Korsmeyer-Peppas equation as plots showed highest linearity with $r^2$ of 0.9939 (Fig. 5) and the value of $n$ was 0.483 (Table 9) concluding that it followed anomalous transport. Optimized formulation remained floated for more than 16 h in vitro. Therefore; in addition to the drug release control for an extended duration, the optimized formulation also showed the excellent floating potential that is the prerequisite for prolonged gastric residence of the formulation. Furthermore, in vivo and pharmacokinetic studies are required to be carried out.

CONCLUSION

This study examines the preparation of a hydrodynamically balanced system containing venlafaxine HCl using the synthetic polymers HPMC K15M as low density hydrophilic polymer, EC and CAP as release modifiers. A systematic study using a Box-Behnken statistical design revealed the most suitable amount of HPMC K15M, EC and CAP in the hydrodynamically balanced system. The optimized formulation fulfilled all the requirements of the target set and showed suitable values of cumulative percentage drug release at 2 h, 8 h, 12 h and total floating time. The drug release pattern followed Korsmeyer-Peppas model with anomalous transport mechanism. The applicability of the statistical optimization techniques in predicting the composition of an optimized formulation is clearly indicated by the present research. It can also be concluded that the hydrodynamically balanced system of venlafaxine HCl can be successfully formulated as an approach to increase the gastric residence time and hence improving its bioavailability. Indeed, this approach might be a better alternative to the conventional dosage form. However, clinical studies should be conducted with optimized formulation in order to correlate the in vitro and in vivo performance.

Table 9. Drug release kinetics of the optimized formulation.

<table>
<thead>
<tr>
<th>No.</th>
<th>Kinetic model</th>
<th>Value of constant</th>
<th>Value of $r^2$</th>
<th>Value of $n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zero-order</td>
<td>$K_0 = 8.181$</td>
<td>0.4723</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>First-order</td>
<td>$K_{1st} = 0.156$</td>
<td>0.9392</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Higuchi</td>
<td>$K_H = 24.142$</td>
<td>0.9930</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Hixson-Crowell</td>
<td>$K_{HC} = 0.043$</td>
<td>0.8638</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Korsmeyer-Peppas</td>
<td>$K_{KP} = 25.007$</td>
<td>0.9939</td>
<td>0.483</td>
</tr>
</tbody>
</table>

Figure 8. Contour plot showing effect of HPMC K15M, EC and CAP on desirability factor. Where HPMC: hydroxypropylmethyl cellulose, EC: ethyl cellulose and CAP: cellulose acetate phthalate. The amounts of HPMC, EC and CAP are in mg.
REFERENCES


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APPLICATION OF LAPLACE TRANSFORMS TO A PHARMACOKINETIC OPEN TWO-COMPARTMENT BODY MODEL

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Abstract: The anti-Laplace of complicated transforms for two-compartment differential equations may be found only in an extensive table of Laplace transforms which usually are not available. Therefore, a general partial fraction theorem was used for obtaining their inverse Laplace transforms. First, the disposition function of the central compartment in a linear two-compartment mammillary model, \( d_{s,c} \), must be written down. Second, except for that disposition function appropriate intake functions were considered with intravenous instantaneous bolus injection, constant zero-order rate infusion, and intramuscular first-order single dose injection of indobufen (Ibustrin). The product of the disposition function and the appropriate input function yields the experimental Laplace transforms for the amount (concentrations) of drug in the central compartment, \( a_{c} \) (\( X_{c} \)).

The input functions for the above mentioned routes of drug administration are equal: \( X_{0} \) (the dose), \( k_{0} \cdot (1 - e^{-\lambda_{i}})/\lambda_{i} \), and \( k_{a} \cdot F \cdot X_{0} / (s + k_{a}) \), respectively. The equations derived are also illustrated in four figures.

Keywords: two-compartment body model, Laplace transforms, the inverse Laplace, partial fraction theorem, disposition function, different intake function: bolus injection, infusion, intramuscular injection

The use of Laplace transforms of a function, \( F(t) \), and the inverse operation, \( L^{-1} \), may be applied to obtain solutions to the systems of linear differential equations of the first-order. However, sufficient matrix algebra is needed to use the above transforms (1). That operation transforms differentiation into multiplication as well as integration into division (1). The Laplace transform enables complex rate expressions to be manipulated easily by conventional algebraic techniques (2). However, it is true with regards to a one-compartment body model. The anti-Laplace of the resulting complicated transforms may be found only in an extensive table of Laplace transforms which usually are not available.

A two-compartment model with intravenous bolus injection

The experimental Laplace transform for the disposition function of the central compartment, \( d_{s,c} \), in a linear two-compartment mammillary model where elimination of drug from central compartment occurs has been provided (2-4):

\[
d_{s,c} = \frac{\Pi(s + E_{1})}{\Pi(s + \lambda_{1})}
\]

where: \( E_{1} = k_{10} + k_{12} \) and \( E_{2} = k_{21} \), and \( \lambda_{1} \) is a disposition rate constant, which may be expressed in terms of the above individual intercompartmental transfer rate constants and elimination rate constants, \( \Pi \) – continued product, \( s \) – the Laplace operator which replaces the time domain of a rate equation.

The model concerned is presented below:

\[
\begin{align*}
    &k_{12} \quad X_{c} \leftrightarrow X_{t} \\
    &k_{21} \quad \downarrow k_{10}
\end{align*}
\]

where \( X_{c} \) and \( X_{t} \) are the amounts of a drug as a function of time in central and tissue compartments, and \( k_{12}, k_{21}, \) and \( k_{10} \) are the first-order rate constants of
transfer a drug from the central compartment to the tissue compartment and vice versa as well as elimination rate constant from the central compartment, respectively. The product of the input, input, and disposition functions yields the Laplace transform for the amount of drug in the central compartment, \( a_{SC} \),

\[
a_{SC} = \text{ins} \cdot d_{SC}
\]

where \( \text{ins} = X_0 \) (a single dose injected) for the above model.

Therefore

\[
\Pi_i = \frac{\tau}{s + E_i} \cdot \frac{\tau}{s + \lambda_i}
\]

The above equation may be rewritten for the central compartment amounts of drug following intravenous bolus injection

\[
a_{SC} = X_0 \cdot \frac{\tau}{s + E_i} \cdot \frac{\tau}{s + \lambda_i}
\]

The anti-Laplace of the resulting transform, \( L^{-1} \) may be obtained by use of a general partial fraction theorem. If the quotient of two polynomials \( P(s)/Q(s) \) is given as above, then

\[
L^{-1} \left( \frac{P(s)}{Q(s)} \right) = \sum_{i=1}^{N} \frac{P(\lambda_i)}{Q(\lambda_i)} \cdot e^{\lambda_i t}
\]

The roots of the polynomial, \( Q(s) \) are \( \lambda_1 = -\alpha \) and \( \lambda_2 = -\beta \). The term \( Q(\lambda) \) may be defined as follows.

When

\[
i = 1 \quad Q(\lambda) = (\lambda + \beta) = (\beta - \alpha)
i = 2 \quad Q(\lambda) = (\lambda + \alpha) = (\alpha - \beta)
\]

Hence the solution for the amount of drug in the central compartment, \( X_C \), applying the general partial fraction equation, is obtained

\[
X_C = X_0 \cdot \sum_{i=1}^{N} \frac{P(\lambda_i)}{Q(\lambda_i)} \cdot e^{\lambda_i t}
\]

A plot of the logarithms of drug plasma concentrations (\( C = X_C/V_d \)) versus time according to the biexponential equation and the method of residuals (2, 6).

Figure 1. Semilog plot of plasma levels vs. time after intravenous bolus administration of the iodine salt to a healthy volunteer described by biexponential equation and the method of residuals (2, 6)
above equation will yield a biexponential curve (Fig. 1).

The disposition constants $\alpha$ and $\beta$ may be obtained applying the method of residuals (Fig. 1).

A two-compartment model with a first-order input process

For a drug that enters the body by an apparent first-order absorption process (generally via the oral or intramuscular routes) and distributes in the body according to a two-compartment model:

$$
X_a \xrightarrow{k_{12}} X_c \leftrightarrow X_T \xleftarrow{k_{21}} X_T
$$

where: $X_a$ – amount of drug at an extravascular site of absorption as a function of time, $k_a$ – the first-order rate constant of absorption.

The disposition function for the central compartment is identical to the disposition function for an intravenous bolus injection

$$
da_{c,c} = \frac{\hat{H}(s + E_i)}{\hat{H}(s + \lambda_i)}
$$

However, the input function is different to describe first-order absorption (2, 5, 6)

$$
in_t = \frac{k_a \cdot F \cdot X_0}{s + k_a}
$$

where $F$ determines the fraction of drug absorbed.

The Laplace transform for the amount of drug in the central compartment equals the product of the disposition and input functions

$$
a_{c,c} = \frac{k_a \cdot F \cdot X_0 \cdot \hat{H}(s + E_i)}{(s + k_a) \cdot \hat{H}(s + \lambda_i)}
$$

Solving for the amount of drug in the central compartment by taking anti-Laplace yields

$$
X_c = k_a \cdot F \cdot X_0 \cdot \frac{\hat{H}(E_i - k_a)}{\hat{H}(\lambda_i - k_a)} \cdot e^{k_a \cdot t} +
+ k_a \cdot F \cdot X_0 \cdot \frac{\hat{H}(E_i - k_a)}{\hat{H}(\lambda_i - k_a)} \cdot e^{\lambda_i \cdot t}
$$

When the hybrid rate constants $\alpha$ and $\beta$ (1) as well as the other constants $B$, $\alpha$, and $C_0$ – the corresponding zero-time intercepts obtained by the method of residuals, are substituted, respectively, the equation may be transformed to a simpler form:

$$
X_c = k_a \cdot F \cdot X_0 \cdot \frac{\hat{H}(E_i - k_a)}{\hat{H}(\lambda_i - k_a)} \cdot (\alpha - \beta) \cdot \cdot e^{\alpha \cdot t} +
+ k_a \cdot F \cdot X_0 \cdot \frac{\hat{H}(E_i - k_a)}{\hat{H}(\lambda_i - k_a)} \cdot (\beta - k_a) \cdot e^{\beta \cdot t}
$$

The plots of the above equation presented on both a graph and on a semilog papers are very char-
Figure 3. Semilog plot of serum (-)-R-indobufen enantiomer vs. time after single 200 mg intramuscular dose administration of racemic indobufen (Ibustrin) to a healthy volunteer described by TopFit 2.0 computer program for an open two-compartment body model (6, 7).

Figure 4. Semilog plot of plasma levels vs. time of a drug that confers two-compartment model characteristics, following constant rate intravenous infusion to steady-state (— — — ) and following the rapid intravenous injection of a dose that gives an initial drug concentration equal to the steady-state concentration (— — — — ) (2).
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A two-compartment model for an intravenous infusion

\[
k_0 \rightarrow X_C \leftrightarrow X_T
\]

\[
k_{12} \quad k_{10}
\]

where: \( k_0 \) – the zero-order rate of drug infusion (constant).

The disposition function for the central compartment is identical to the disposition function for an intravenous bolus injection.

\[
d_{s,c} = \frac{s + E_2}{(s + \lambda_1) \cdot (s + \lambda_2)}
\]  

\[ (14) \]

Multiplication of this disposition function by the input function for an intravenous infusion beginning at time zero (i.e., \( in_s = k_0 \cdot (1 - e^{-\lambda t})/s \)) yields the following Laplace transform for the amount of drug in the central compartment

\[
a_{s,c} = \frac{k_0 \cdot (s + E_2) \cdot (1 - e^{-\lambda t})}{s \cdot (s + \lambda_1) \cdot (s + \lambda_2)}
\]  

where \( T \) is the duration of an infusion.

The above two polynomials fulfill the requirements for the use of a partial fraction theorem for obtaining inverse Laplace transforms. Hence the solution for the amount of drug in the central compartment \( XC \) as a function of time may be written

\[
X_c = \frac{k_0 \cdot (\alpha - k_{21}) \cdot (e^{-\alpha t} - e^{-\beta t})}{\alpha \cdot (\alpha - \beta)} + \frac{k_0 \cdot (\beta - k_{31}) \cdot (e^{-\beta t} - e^{-\alpha t})}{\beta \cdot (\beta - \alpha)}
\]  

\[ (18) \]

It should be noted that the above single equation describes the amount of drug in the central compartment as a function of time while the infusion is being carried out and after infusion stops. While the infusion is continuing, \( T = t \) and varies with time

\[
X_c = \frac{k_0 \cdot (\alpha - k_{21}) \cdot (e^{-\alpha t} - e^{-\beta t})}{\alpha \cdot (\alpha - \beta)} + \frac{k_0 \cdot (\beta - k_{31}) \cdot (e^{-\beta t} - e^{-\alpha t})}{\beta \cdot (\beta - \alpha)}
\]  

However, when infusion ceases, time \( t \) becomes a constant corresponding to \( T \) – the duration of the infusion. It should be apparent that upon stopping the infusion, drug concentrations in the plasma decline in a biexponential manner (Fig. 4).

It can be seen from Fig. 4 that the biexponential characteristics of the drug is more evident following the bolus injection than after terminating the infusion.

Acknowledgment

This publication has been written to give thanks to the Almighty God for my 80th birthday anniversary which hopefully I am supposed to survive on June 13, 2017.

REFERENCES


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FORMULATION AND CHARACTERIZATION OF LORATADINE CONTAINING ORODISPERSIBLE LYOPHILIZATES AND FILMS

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Abstract: Orodispersible formulations are relatively novel solid dosage forms that involve rapid disintegration or dissolution in the mouth. They enable an easy application and originally were developed to address swallowing difficulties of conventional tablets and capsules. The aim of this study was to develop and quality evaluate oral lyophilizates and orodispersible films with loratadine. Based on the preliminary studies, optimal composition of lyophilizates and films were chosen for further studies. Oral lyophilizates were obtained by freeze-drying process using different concentration of gelatin, mannitol and sodium bicarbonate. Films were prepared by solvent-casting method with different concentrations of hypromellose and glycerol used as plasticizer. Obtained formulations were characterized for drug content, disintegration time, organoleptic and mechanical properties. In order to identify surface morphology of obtained solid dosage forms, scanning electron microscopy was used. The results showed that designed dosage forms with loratadine were characterized by appropriate mechanical properties, the uniform content of the drug substance and low moisture content. Disintegration time measured in vivo was = 36 s for films and = 9 s for lyophilizates.

Keywords: oral lyophilizates, orodispersible films, loratadine, solvent-casting method, freeze-drying method

Oral route is the most popular and preferred method of drug application (1-3). Orodispersible films and oral lyophilizates are relatively novel solid dosage form which placed in the mouth quickly dissolve or disintegrate in the saliva without necessity of drinking water. Their advantages include no risk of choking, easier acceptance and dosage in comparison with conventional solid forms of the drug (4-7). Different manufacturing technologies of orodispersible forms have been developed. Lyophilizates can be prepared by freeze-drying, while films are obtained by solvent casting, hot-melt extrusion, sublimation and compression methods (8-13). Freeze-drying involves freezing of solution, suspension or emulsion containing drug with excipients followed by sublimation of ice. Rapid cooling results in freezing the system with simultaneous crystallization of ice in the form of fine crystals, which sublimate at reduced pressure, forming porous tablet matrix. Due to high porosity, oral lyophilizates disperse in the oral cavity within a few seconds (14-16). In the solvent casting method, the active substance, polymer and excipients are dissolved or suspended in water or in a mixture of water/organic solvents. The prepared mass is then poured into glass forms followed by solvent evaporation. The obtained films are further divided into pieces with the size corresponding to a proper dosage of drug (17, 18).

The main component in oral lyophilizates is gelatin, while in orodispersible films – polymer or a mixture of polymers which provide optimal elasticity. Gelatin is a mixture of purified protein fractions obtained by acid hydrolysis of animal origin collagen. It provides a uniform dispersion of the drug substances and does not increase the disintegration time of obtained lyophilizates (17). Polymers often used in films production include pullulan, povidone, chitosan or hypromellose (Pharmacoat® 606, Methocel®, Metolose®) (18). Pharmacoat® 606 is hydrophilic polymer forming transparent, odorless and tasteless films. It is a non-ionic cellulose ether with an average molecular weight from 10 000 to 150 000 Da (19).

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Loratadine is a second-generation histamine H<sub>1</sub> receptor antagonist commonly used in the treatment of allergic rhinitis and urticaria. It is a white, crystalline powder not soluble in water. Loratadine is a good candidate for fast disintegrating oral preparations, because it is characterized by an acceptable taste and it does not require taste masking (20, 21).

The aim of this work was to design and characterize oral lyophilizates and orodispersible films with loratadine. The films were obtained by casting-solvent method using Pharmacoat® 606 and glycerol, while lyophilizates were formulated by freeze-drying process using different concentration of gelatin, mannitol, and sodium bicarbonate. The obtained dosage forms were characterized for uniformity of weight and thickness, moisture content and drug loading. Disintegration time of the films and lyophilizates were measured in vivo and in vitro by two independent methods. The morphology of formulations was determined by scanning electron microscopy. The sensory evaluation in vivo was also performed. Mechanical properties of the films were determined by using TA.XT. Plus Texture Analyzer and expressed by three different parameters: percent of elongation, tensile strength and Young’s modulus. Additionally, film folding endurance was examined.

**EXPERIMENTAL**

**Materials**

Hypermellose (Pharmacoat® 606) was obtained from ShinEtsu Chemical, Tokyo, Japan. Polyethylene glycol was purchased from Donauchem, Warsaw, Poland. Gelatin (type B), sodium bicarbonate, mannitol and glycerol were purchased from Sigma Aldrich, Steinheim, Germany. Water was distilled and passed through a reverse osmosis system Milli-Q Reagent Water System (Billerica, MA, USA). PCV blisters (diameter of 13 mm and depth of 5 mm) were obtained from Fagron, Kraków, Poland.

**Methods**

**Preparation of oral lyophilizates**

Oral lyophilizates were obtained by the freeze-drying method. Gelatin was dissolved in distilled water at about 40°C to obtain concentrations of 0.5 and 1%. Different amounts of mannitol (2.5, 5.0, 10.0 and 20.0%) and sodium bicarbonate (0.25 and 0.5%) were added to gelatin solution (Table 1).

Then, loratadine was suspended in a solution so that the amount of 0.5 g of final suspension contained 10 mg of drug. Obtained suspensions were dosed into PCV blisters (0.5 g into each blister slot), frozen at -20°C for 10 min and freeze-dried (lyophilizer FreeZone System, Labconco, Kansas, MO, USA). In order to set the optimal parameters of the freeze-drying process to obtain product of the desired properties, a number of preliminary tests were conducted and the experimental parameters were chosen. Experiments were carried out under the following conditions: primary drying for 4 h at -48°C, pressure of 0.08 mbar with gradually increasing the temperature to 20°C. In the second drying step, the temperature was set to 20°C for 1 h and vacuum pressure of 0.08 mbar. After preliminary studies, formulations (L2, L3, L7 and L8) with short disintegration time and optimal mechanical strength (these formulations did not crumble and were easily removed from the blister) were prepared with loratadine (L2L, L3L, L7L and L8L).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Gelatin (g)</th>
<th>Mannitol (g)</th>
<th>Sodium bicarbonate (g)</th>
<th>Water (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>2.5</td>
<td>0.25</td>
<td></td>
<td>up to 100.0</td>
</tr>
<tr>
<td>L2</td>
<td>1.0</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>10.0</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>5.0</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>20.0</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L6</td>
<td>1.0</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L7</td>
<td>5.0</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L8</td>
<td>10.0</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L9</td>
<td>5.0</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L10</td>
<td>20.0</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Preparation of orodispersible films

Films were prepared by the solvent casting method. Film forming agent (Pharmacoat® 606) was used in three concentrations: 2.5%, 5% and 10%. Effect of plasticizer type (glycerol, polyethylene glycol and mixture of both) with different concentration was additionally evaluated. An overview on film composition is given in Table 2. After preliminary studies, formulations (F1, F2, F3), which formed smooth, non-sticky, fast disintegrating and easily peelable films were selected for loratadine loaded films preparation (F1L, F2L and F3L). Pharmacoat® 606 was dissolved in distilled water at temperature of 50°C. Glycerol was dissolved in the polymer solution and homogenized 1 h using a magnetic stirrer. After that, 2 g of loratadine was added. Obtained suspensions were casted onto a Petri dish and dried at temperature room for 24 h. After drying, films were cut into rectangular pieces of 2 cm × 3 cm, which contained 10 mg of loratadine each.

Evaluation of oral lyophilizates and orodispersible films

Uniformity of weight and thickness

Weight and thickness of obtained formulations were evaluated according to the specifications of European Pharmacopoeia (22). Film thickness was measured by a micrometer (precision ± 0.001 mm, Helios-preisser, Gammertingen, Germany) at three
different positions on the film. Lyophilizates thickness was measured using calibrated digital caliper (Beta 1651 DGT, Milan, Italy). Films and lyophilizates were weighed individually using an electronic balance (XA 60/220, Radwag, Radom, Poland).

**Drug content**

Loratadine content uniformity in films and lyophilizates was tested by dissolving the dosage form in 10 mL of 0.1 M HCl. Then, 1 mL of the solution was withdrawn and diluted by a mobile phase. After filtration through 0.45 µm cellulose acetate filters (Millipore, Billerica, MA, USA), concentration of loratadine was determined by the HPLC system Agilent Technologies 1200 equipped with a G1312A binary pump, a G1316A thermostat, a G1379B degasser and a G1315B diode array detector (Agilent, Waldbronn, Germany). Data collection and analysis were performed using Chemstation 6.0 software. Isocratic separation was achieved on a Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 µm column (Agilent, Waldbronn, Germany). Mobile phase was 0.025 M sodium phosphate buffer pH 3.7 and acetonitrile (1 : 4; v/v), the flow rate was 1.0 mL/min and UV detection was performed at a wavelength of 247 nm (23-26). For injection into the HPLC system, 20 µL of sample was used. The retention time of loratadine was 3.5 min. Standard calibration curve was linear over the range of 1 – 100 µg/mL with the correlation coefficient (R²) 0.999.

**Sensory evaluation**

Sensory evaluation of roughness and taste of dosage forms was carried out by six healthy volunteers (Research Ethics Committee at Medical University of Białystok approval number R-J-002/262/2014). Before the assay, potential probants were tested by the sensitivity of taste. For this reason, basic taste solutions with different concentrations of model substances: sour (tartaric acid), sweet (sucrose), salty (sodium chloride) and bitter (quinine hydrochloride) in distilled water were prepared. For further studies volunteers with the highest taste sensitivity were selected (27). A numerical scale was used with the following values: 0 – pleasure/not rough; 1 – slightly pleasure/slightly rough; and 2 – unpleasure/rough.

**Morphology analysis**

Morphology analysis was performed using scanning electron microscope (Hitachi S4200, Tokyo, Japan). Samples were sputter-coated with gold before imaging.

**Evaluation of disintegration time**

**In vivo**

Disintegration time of the lyophilizates and films in the oral cavity was evaluated by six healthy volunteers. The study protocol was approved by the Research Ethics Committee at the Medical University of Białystok (number R-J-002/262/2014) and complied with the principles of the Declaration of Helsinki. After the mouth was rinsed with purified water, lyophilizate or film was hold in the mouth without chewing until the dosage form disintegrated. The time required for the complete disintegration in the oral cavity was noted.

**In conventional disintegration apparatus**

Disintegration time of formulations was measured using conventional apparatus (Erweka ED-2L, Heusenstamm, Germany) according to European Pharmacopoeia (22). Distilled water was used as disintegration medium.

**On Petri dish**

Petri dish with a diameter of 7 cm was filled with 7 mL of water and the lyophilizate or film (n = 6) was carefully put in the center. Time needed to completely disintegrate into fine particles was measured (28).

**Moisture content**

Content of moisture in films and lyophilizates was assessed using moisture analyzer balance (Radwag WSP 50SX, Radom, Poland).

**Evaluation of mechanical film properties**

Mechanical properties were examined using Texture Analyzer TA.XT. Plus (Stable Microsystems, Godalming, UK) and expressed by three different parameters – percent of elongation (E%), tensile strength (TS) and Young’s modulus (E). Experimental parameters of the process were chosen during preliminary tests and set as follows: pre-test speed 1.00 mm/s, test speed 1.00 mm/s, post-test speed 1.00 mm/s, distance 3.0 mm, strain 10.0%, trigger force 0.001 N, break sensitivity 0.107 N. TS was measured by applied stress and calculated by dividing the force per area. The E% and E were calculated by following equations:

$$E\% = \frac{L - L_o}{L_o} \times 100$$

$$E = \frac{\delta}{\varepsilon}$$

where: $L_o$ – length of the sample prior to the measurement, $L$ – length of the sample after the measurement, $\delta$ – tension, $\varepsilon$ – linear deformation (29).
Additionally, film folding endurance was examined and the number of folds necessary to break the film was defined (30).

**RESULTS AND DISCUSSION**

The choice of the excipients in the design of dosage forms is critical because it influences their properties and quality. The short disintegration time and appropriate mechanical strength are crucial factors in orodispersible delivery systems. The properties of orodispersible films and oral lyophilizates mainly depend on: amount and type of polymer, matrix forming agent, amount of solvents, and type of manufacturing process. In the case of films, type and amount of plasticizer is also important (2-5). In order to determine the optimal composition, various excipients in different amounts have been examined. For this purpose, 10 different formulations of oral lyophilizates (Table 1) and 27 of orodispersible film formulations (Table 2) were designed and examined.

In order to develop a composition of oral lyophilizates, the effect of gelatin, mannitol and sodium bicarbonate was tested. Based on the placebo lyophilizates evaluation, it was shown that lower concentration of mannitol and higher concentration of sodium bicarbonate resulted in too crumbly, not durable texture. Interestingly, there were no significant differences in disintegration time between formulations prepared with various gelatin concentrations (data not shown). All placebo lyophilizates had a very low moisture content (from 0.2% in L2 to 1% in L3). Lyophilizates containing mannitol as a filler at the amount of 5 and 10% and with lower (0.25%) amount of sodium bicarbonate (L2, L3, L7, L8) were characterized by sufficient mechanical properties, which are very important in further processes such as packaging, opening and removing the drug from the packaging, thus they were selected for further studies with loratadine. Lyophilizates with the addition of 0.5% of sodium bicarbonate as disintegrating agent were crumbly and it was impossible to remove them from blisters.

### Table 3. Physicochemical characteristics of orodispersible films and oral lyophilizates with loratadine.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Weight (mg)</th>
<th>Thickness (µm/mm)</th>
<th>Drug content (mg)</th>
<th>Moisture content (%)</th>
<th>Disintegrating time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Films</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1L</td>
<td>242.0 ± 2.0</td>
<td>42.0 ± 2 µm</td>
<td>10.0 ± 1.0</td>
<td>0.35 ± 0.2</td>
<td>34.0 ± 1.0, 30.0 ± 1.2, 38.0 ± 1.1</td>
</tr>
<tr>
<td>F2L</td>
<td>269.0 ± 1.8</td>
<td>44.0 ± 1 µm</td>
<td>9.8 ± 0.8</td>
<td>0.72 ± 0.1</td>
<td>34.0 ± 0.9, 30.0 ± 1.0, 37.0 ± 1.2</td>
</tr>
<tr>
<td>F3L</td>
<td>312.0 ± 1.2</td>
<td>48.0 ± 3 µm</td>
<td>10.3 ± 0.5</td>
<td>1.0 ± 0.2</td>
<td>36.0 ± 1.2, 31.0 ± 0.9, 40.0 ± 1.5</td>
</tr>
<tr>
<td><strong>Lyophilizates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2L</td>
<td>63.7 ± 0.9</td>
<td>2.0 mm</td>
<td>9.93 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>9.0 ± 0.5, 6.0 ± 0.5, 9.0 ± 0.6</td>
</tr>
<tr>
<td>L3L</td>
<td>63.2 ± 1.1</td>
<td>2.0 mm</td>
<td>8.75 ± 0.6</td>
<td>0.75 ± 0.2</td>
<td>9.0 ± 0.6, 6.0 ± 0.6, 7.0 ± 0.5</td>
</tr>
<tr>
<td>L7L</td>
<td>64.3 ± 1.2</td>
<td>2.0 mm</td>
<td>10.73 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>8.0 ± 0.5, 6.0 ± 0.6, 6.0 ± 0.5</td>
</tr>
<tr>
<td>L8L</td>
<td>64.4 ± 0.8</td>
<td>2.0 mm</td>
<td>9.3 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>8.0 ± 0.5, 6.0 ± 0.5, 6.0 ± 0.5</td>
</tr>
</tbody>
</table>

### Table 4. Sensory evaluation of designed orodispersible films and oral lyophilizates with loratadine.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Taste/Roughness a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1L</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Scored as follows: 0 = pleasure/not rough; 1 = slightly pleasure/slightly rough; 2 = unpleasure/rough
To choose optimal excipients and their concentrations for placebo orodispersible films, the effect of polyethylene glycol, glycerol and Pharmacoat® 606 on their physico-chemical properties have been examined. Disintegration time of films was mainly influenced by the polymer concentration. With the increased amount of polymer, increased disintegration time was observed. The shortest disintegration time was noted in formulations containing 2.5% Pharmacoat 606®, therefore this concentration of polymer was used to preparing films with loratadine. Films containing glycerol as plasticizer possessed better mechanical strength compared to formulations with polyethylene glycol. Films with polyethylene glycol addition were characterized by greater moisture content and tended to stick together. Moreover, volunteers claimed that films with the addition of polyethylene glycol or polyethylene glycol/glycerol mixture were characterized by unpleasant taste. Interestingly, mixture of both plasticizers did not improve the mechanical properties of films (data not shown). Films placebo F1, F2, and F3 were chosen for preparing formulations with loratadine.

Characteristics of films and lyophilizates with loratadine is shown in Table 3. Thickness uniformity is extremely important as it directly influences dosage of drug. Thickness of lyophilizates was 2 mm and for orodispersible films it ranged from 42.0 to 48.0 µm. Loratadine loading was uniform (22) in all formulations (for orodispersible films, it was in the range between 9.8 and 10.3 mg and for lyophilizates – from 8.75 to 10.73 mg). All prepared films possessed low moisture content (0.35 – 1%), did no stick and showed no blooming.

Disintegration time is crucial feature of orodispersible dosage forms and depends on the volume of medium used (31, 32). As volume of saliva in the oral cavity is less than 7 mL, therefore it is really important to measure disintegration time not only in the conventional pharmacopoeial apparatus. Petri
Figure 2. Mechanical properties of orodispersible films with loratadine expressed as percent at elongation (A), tensile strength (B), Young’s modulus (C) and folding endurance (D).
dish with 7 mL of water was used as an alternative method to evaluate the in vitro disintegration rate. The values of disintegration time measured by conventional apparatus for films and lyophilizates were $= 31$ s and 6 s, respectively. Disintegration time evaluated in vivo for films was $= 36$ s and for lyophilizates $= 9$ s.

Obtained lyophilizates were characterized by porous structure (Fig. 1 A, B), which allows penetration of saliva into the matrix, resulting in rapid disintegration. In the surface analysis of films, cohesive and homogeneous matrix was observed. Absence of pores and surface uniformity depicts good quality of films (Fig. 1 C, D). Moreover, the structure of films and lyophilizates showed regular arrangement of loratadine crystals (Fig. 1 B, D).

Organoleptic properties like taste, mouth-feeling and roughness are of considerable importance in orally disintegrating dosage forms design, therefore the sensory evaluation was also performed. Prepared films and lyophilizates were characterized by very pleasant taste and no roughness was reported (Table 4).

Mechanical properties of films containing loratadine expressed by three different parameters – percent at elongation (E%), tensile strength (TS) and Young’s modulus (E) are presented in Figure 2 A-C. Tensile strength (TS) is defined as maximum stress applied at which the film breaks and it assesses the mechanical strength of films. Young’s modulus is the measure of film stiffness (32-34). Elastic and flexible film is characterized by low value of TS and high value of percent of elongation. These parameters are very important during removal, cutting, packaging, transport and storage of films (35). Mechanical properties such as tensile strength and percent of elongation are influenced by plasticizer added to the formulation (32, 36). It was shown that glycerol as plasticizer in combination with Pharmacoat® 606 as membrane-forming substance ensured appropriate mechanical properties – obtained films were transparent, elastic and flexible. Folding endurance is another parameter determining the mechanical properties of a film. It is measured by repeatedly folding a film at the same point until it breaks. Folding endurance value is a number of times the film is folded without breaking. Higher folding endurance value depicts more mechanical strength of a film. As mechanical strength is governed by plasticizer concentrations, it is clearly evident that plasticizer concentration also indirectly affects folding endurance value (34, 36). For formulation F1L folding endurance was 61, for F2L it was 90, and for formulation containing the highest amount of plasticizer – F3L, folding endurance was 100 (Fig. 2 D).

CONCLUSIONS

In this work, formulation of loratadine in orodispersible films and oral lyophilizates was achieved. Obtained dosage forms possessed very short disintegration time evaluated in vivo ($= 36$ s for films and $= 9$ s for lyophilizates). All formulations were characterized by uniformity of the drug content, weight and thickness. Prepared films and lyophilizates with loratadine showed pleasant taste and mouth feeling which is very important for orally administrated products. Glycerol as plasticizer in combination with Pharmacoat® 606 ensured appropriate mechanical properties – films were transparent, elastic and flexible. Designed formulations are supposed to be promising alternative for conventional solid dosage forms with loratadine.

REFERENCES


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Hypertension is the prominent cause for stroke, cardiovascular disorder and renal disease which further lead to death of an individual. It has been forecast that there will be a relative increase of about 24% in the occurrence of hypertension in the developed countries from 2000 to 2025 (1). Karen et al., in their study surveyed that the number of adults with hypertension has doubled from 1995 to 2005 (2). So the treatment of hypertension has become the foremost requirement to have a control on increasing death rate. Lowering blood pressure is the leading treatment to ensure a downfall in the risk of cardiovascular morbidity and mortality. The renin-angiotensin system plays a commanding role in blood pressure regulation and is the major target for many antihypertensive drugs like olmesartan medoxomil (OLM) which is a selective AT1 subtype angiotensin II receptor antagonists and is approved for effective treatment of hypertension, kidney disease and heart failure (3, 4). Clinical trials in hypertensive patients with OLM revealed tremendous pharmacological actions and a good tolerance without any serious side effect (5). Moreover, the efficiency of OLM has been revealed to be much higher or at least equivalent to many other commonly prescribed antihypertensive agents (6). It is also a promising drug for diminishing the vascular risk in high-risk elderly patients with new-onset diabetes. It is commercially available as conventional tablets in the strength of 10 and 20 mg. The maximum dose in the treatment of hypertension is 40 mg (7).

However, these marketed formulations of OLM are available in crystalline form which is having low solubility and dissolution rate. One of its commercial tablet formulations under the brand name “Benicar” possesses oral bioavailability of only 26% in healthy humans (8). Even the remaining unabsorbed drug leads to gastrointestinal side effects such as dyspepsia, abdominal pain, gastroenteritis and nausea. This reduced oral bioavailability of OLM is due to its low aqueous solubility (< 7.75 µg/mL) (9).

EFFECT OF POLYMER AND METHOD ON PARTICLE SIZE AND CRYSTALLINITY OF OLMESARTAN MEDOXOMIL DURING THE FORMULATION OF SOLID DISPERSIONS

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Abstract: This study explores the use of modified locust bean gum and poloxamer 188 as carriers to improve the dissolution rate of olmesartan medoxomil; whilst also investigating the effect of technique (lyophilization and modified solvent evaporation) used for the preparation of solid dispersions. Both carriers along with methods showed significant variability in particle size and, in turn, the solubility profile of olmesartan medoxomil indicating the great influence of carrier/method used. The solid dispersion prepared with lyophilization method using modified locust bean gum as a carrier showed reduced particle size of 58.8 ± 1.8 nm as indicated by particle size analysis resulting in higher solubility (1097 ± 15.92 µg/mL) and dissolution efficiency (84.39% after 120 min) as compared to other carrier and method. The resulting solid dispersions were characterized using X-ray diffraction (XRD), differential scanning calorimetry (DSC), scanning electron microscopy (SEM) and transmission electron microscopy (TEM), which further supported the findings. As indicated by TEM, optimized formulation showed spherical and reduced particles of around 34-36 nm which may have contributed to the improved dissolution rate of drug. The optimized solid dispersion was then formulated into fast dissolving tablet to achieve instantaneous and enhanced release for the better and immediate treatment of hypertension.

Keywords: solubility, freeze drying, particle size, fast dissolving tablet, modified locust bean gum

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Improvement in oral bioavailability of OLM can augment its clinical efficacy and can reduce the oral dose required to achieve equivalent effect and, in turn, diminish the side effects. This oral bioavailability of poorly water soluble drug can be increased by increasing its solubility. Various techniques such as complex formation (10), solid dispersion (11-13), liquisolid compact (14), nanosuspension (8), nanoemulsion (15) and self-nanoemulsifying drug delivery systems (16) have been reported for improving the solubility of OLM. Many authors have classed the solid dispersion technique as one of the most effective and economical method for improving dissolution rate (17, 18) and has been widely explored for enhancing the solubility and, in turn, efficacy of many pharmaceutical agents viz. ezetimibe (19), tacrolimus (20), meloxicam (21), tovaptan (22), etc. The fact behind the enhancement in solubility attained by the formation of solid dispersions using hydrophilic carrier is the reduction in particle size, amorphization of a crystalline drug, reduced agglomeration, increased wet ability and molecular dispersibility. The prominent effect of particle size reduction on the drug dissolution rate has not been studied so far for any drug formulated as solid dispersion. According to Freundlich–Ostwald equation, the reduction of particle size into nanometric range enhances dissolution rate of drug not only due to increased surface area but also due to increased saturation solubility (23).

The aim of the present study is to enhance the solubility and dissolution rate of OLM and compare the potential of a natural (modified locust bean gum) and synthetic (poloxamer 188) carrier along with the method (lyophilization and modified solvent evaporation) for the preparation of solid dispersions and also to determine the effect of individual carrier/method on particle size of solid dispersions. This might assist the future research in selecting the most appropriate carrier and method for the development of solid dispersion formulations which is a critical parameter for producing formulations with desired solubility and dissolution profile. The study also relates to the fabrication of optimized formulation into fast dissolving tablet to achieve instantaneous release for the better and immediate treatment of hypertension.

MATERIALS AND METHODS

Olmesartan medoxomil was obtained as a gift sample from Cipla Ltd., Mumbai. Locust bean gum was obtained from Wilson Laboratories, Mumbai. Poloxamer 188 was purchased from Sigma Aldrich, Mumbai. Methanol and ethanol were purchased from S.D. Fine Chemicals, Mumbai. Camphor was purchased from Loba Chem Pvt. Ltd., Mumbai. Crospovidone was purchased from Signet Chemicals Pvt. Ltd., Mumbai. All other chemicals used were of analytical grade.

Preparation of modified locust bean gum

Locust bean gum was modified according to the method described by Murali Mohan Babu et al. (24). For the preparation of modified locust bean gum, powdered locust bean gum was heated in hot air oven (Decibel, Chandigarh, India) at 120°C for 2 h. The prepared modified locust bean gum was then sieved (100 mesh) and stored in an air tight container at 25°C. Both locust bean gum and modified locust bean gum were then characterized for viscosity using Rheometer (Rheolab QC, Anton Paar,

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LBG</th>
<th>MLBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity Shear Rate (s⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1903 ± 26</td>
<td>192.1 ± 1.6</td>
</tr>
<tr>
<td>20</td>
<td>1629 ± 19</td>
<td>169.1 ± 1.9</td>
</tr>
<tr>
<td>30</td>
<td>1414 ± 17</td>
<td>150.9 ± 1.2</td>
</tr>
<tr>
<td>40</td>
<td>1251 ± 13</td>
<td>137.2 ± 1.5</td>
</tr>
<tr>
<td>Swelling index (%)</td>
<td>1494 ± 12</td>
<td>1319 ± 15</td>
</tr>
<tr>
<td>Hydration Capacity (%)</td>
<td>2.9 ± 0.03</td>
<td>2.93 ± 0.02</td>
</tr>
<tr>
<td>Moisture Sorption Capacity (%)</td>
<td>7.4 ± 0.18</td>
<td>12.96 ± 0.21</td>
</tr>
<tr>
<td>Angle of Repose</td>
<td>36.67 ± 0.9</td>
<td>36.29 ± 0.8</td>
</tr>
<tr>
<td>Carr’s Index (%)</td>
<td>21.50 ± 0.7</td>
<td>20.82 ± 0.5</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.D. (n = 3).
* LBG = Locust bean gum, MLBG = Modified Locust bean gum
Table 2. Composition and characterization of prepared solid dispersions of olmesartan medoxomil.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Formulation code</th>
<th>Drug: carrier ratio</th>
<th>Method used</th>
<th>% Yield</th>
<th>% Drug content</th>
<th>Particle size</th>
<th>Saturation solubility (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure Drug</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9 ± 0.2</td>
</tr>
<tr>
<td>PMO1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>66 ± 3.76</td>
</tr>
<tr>
<td>OLM1</td>
<td>1 : 1</td>
<td>Modified solvent evaporation</td>
<td>94.5 ± 1.2</td>
<td>98.4 ± 1.3</td>
<td>974.1 ± 3.3</td>
<td>192 ± 2.45</td>
<td></td>
</tr>
<tr>
<td>OLM2</td>
<td>1 : 2</td>
<td>Modified solvent evaporation</td>
<td>91.7 ± 1.0</td>
<td>97.6 ± 1.0</td>
<td>956.2 ± 3.0</td>
<td>237 ± 5.98</td>
<td></td>
</tr>
<tr>
<td>OLM3</td>
<td>1 : 3</td>
<td>Modified solvent evaporation</td>
<td>82.1 ± 1.5</td>
<td>94.8 ± 1.4</td>
<td>916.3 ± 2.5</td>
<td>389 ± 5.19</td>
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</tr>
<tr>
<td>OLM4</td>
<td>1 : 4</td>
<td>Modified solvent evaporation</td>
<td>73.5 ± 0.9</td>
<td>95.8 ± 1.1</td>
<td>877.3 ± 4.1</td>
<td>557 ± 11.95</td>
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<td>OLM5</td>
<td>1 : 5</td>
<td>Modified solvent evaporation</td>
<td>68.8 ± 0.8</td>
<td>98.6 ± 1.0</td>
<td>871.8 ± 3.2</td>
<td>593 ± 10.48</td>
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<tr>
<td>OLM6</td>
<td>1 : 1</td>
<td>Lyophilisation</td>
<td>92.7 ± 1.3</td>
<td>96.5 ± 0.9</td>
<td>597.6 ± 1.7</td>
<td>358 ± 7.45</td>
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<tr>
<td>OLM7</td>
<td>1 : 2</td>
<td>Lyophilisation</td>
<td>83.1 ± 1.1</td>
<td>99.5 ± 0.8</td>
<td>561.9 ± 3.2</td>
<td>537 ± 6.85</td>
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<td>1 : 3</td>
<td>Lyophilisation</td>
<td>74.1 ± 0.9</td>
<td>96.8 ± 1.1</td>
<td>533.9 ± 2.9</td>
<td>789 ± 7.58</td>
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<td>OLM9</td>
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<td>Lyophilisation</td>
<td>72.5 ± 0.7</td>
<td>97.9 ± 1.2</td>
<td>514.3 ± 2.5</td>
<td>957 ± 13.85</td>
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<tr>
<td>OLM10</td>
<td>1 : 5</td>
<td>Lyophilisation</td>
<td>70.1 ± 0.9</td>
<td>98.1 ± 0.8</td>
<td>515.5 ± 4.5</td>
<td>963 ± 12.57</td>
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<td>PMO2</td>
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<td>79 ± 2.3</td>
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<td>OLM11</td>
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<td>Modified solvent evaporation</td>
<td>94.8 ± 1.1</td>
<td>99.1 ± 1.2</td>
<td>893.9 ± 1.5</td>
<td>278 ± 6.81</td>
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<td>Modified solvent evaporation</td>
<td>92.9 ± 1.0</td>
<td>98.0 ± 1.1</td>
<td>881.7 ± 2.7</td>
<td>399 ± 7.92</td>
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<td>OLM13</td>
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<td>Modified solvent evaporation</td>
<td>94.1 ± 0.6</td>
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<td>96.3 ± 0.9</td>
<td>98.4 ± 1.3</td>
<td>238.6 ± 3.1</td>
<td>649 ± 5.99</td>
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<tr>
<td>OLM15</td>
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<td>769.4 ± 2.1</td>
<td>746 ± 13.46</td>
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<td>297.3 ± 2.1</td>
<td>693 ± 17.64</td>
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<td>93.8 ± 1.9</td>
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<td>58.8 ± 1.8</td>
<td>1097 ± 15.92</td>
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<tr>
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<td>1102 ± 19.51</td>
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Data are expressed as mean ± S.D. (n = 3)
Ostfildern, Germany) at different shear rate. They were also evaluated for their swelling index, hydration capacity, moisture sorption capacity, angle of repose and Carr’s index (24, 25).

Drug excipients compatibility studies
While designing the solid dispersions, it is imperative to give consideration to the compatibility of drug and carrier used within the systems. It is therefore necessary to confirm that the drug is not showing any incompatibility with the carrier under experimental conditions (40 ± 5°C and 75 ± 5% RH) for at least 2 weeks. Desired quantity of drug with specified excipients (poloxamer 188 and modified locust bean gum) in the ratio 1 : 5 was taken and mixed thoroughly, sieved and filled in dried vials. The vials were examined daily at regular intervals for discoloration, clump formation and liquefaction. Also the FTIR spectra of pure drug, carrier and solid dispersion were obtained to determine the compatibility. For FTIR, each sample of approximately 4 mg was kept in FTIR spectrometer (ALPHA FTIR, Bruker, Germany) and spectra were recorded.

Formulation preparation
Modified solvent evaporation method
For the preparation of solid dispersions, the drug was dissolved in suitable quantity of methanol with continuous stirring. Both the carriers (poloxamer 188 and modified locust bean gum) were dissolved separately in appropriate quantity of water (up to the wet mass of carrier). The drug to carrier ratios were varied from 1 : 1 to 1 : 5 to determine the effect of increasing concentration of carrier. The drug solution was then added to the carrier solution to form a clear solution. The entire solvent was evaporated under reduced pressure at 60-70°C using rota evaporator (RV-10, IKA, Bangalore, India). The obtained solid dispersions were dried, sieved and stored in a desiccator (25).

Lyophilization (Freeze drying method)
Solid dispersions containing different drug to carrier ratios from 1 : 1 to 1 : 5 were also prepared by freeze drying method. OLM was dissolved in sufficient quantity of methanol to form phase I. Similarly, carriers were dissolved separately in
water to form phase II and both the phases were mixed together. Methanol was evaporated and resulted solution was freeze-dried in a quick/deep freezer (RQFV-265, REMI Elektrotechnik, Mumbai, India) at -20°C and was then lyophilized in a freeze dryer (LyoQuest-55, Azbil Telstar Technologies, Terrassa, Spain) at a temperature of -45°C and a vacuum of 0.200 Mbar. The freeze dried mass was then sieved through sieve no. 60 and stored in desiccator (26, 27).

Characterization of solid dispersion formulation

Drug content of the prepared solid dispersions was determined by dissolving solid dispersions equivalent to 10 mg of OLM in methanol. The diluted solution was then filtered through membrane filters (Millipore Corp, Billerica, US) of size 0.45 µm and analyzed using high performance liquid chromatography (HPLC) assay (1260 series, Agilent Technology, Santa Clara, CA, USA) with mobile phase consisting of acetonitrile and 0.05 M sodium dihydrogen phosphate in a ratio of 60:40 (28). The mobile phase at flow rate of 1 mL/min was eluted in BDS Hypersil C-18, 150 × 4.6 mm, 5 µ column (Thermo Fisher Scientific Inc., USA) at room temperature and samples were analyzed at 257 nm using UV diode array detector. The retention time of drug was observed at 3.54 ± 0.05 min.

The percentage yield of each formulation was determined according to the recoverable final weight of solid dispersions using following eq.

\[
\text{Percentage yield} = \frac{\text{Practical weight of solid dispersion}}{\text{Theoretical weight of solid dispersion}} 
\]

Solubility of prepared solid dispersions

The solubility of solid dispersions was determined by adding solid dispersions equivalent to 20 mg of drug to 10 mL of triple distilled water in screw capped vials. The vials were sealed and kept on isothermal water bath shaker (NSW-125, Narang Scientific Works, New Delhi, India) at 3 ± 0.5°C for 48 h. Then, the resulted samples were centrifuged and supernatant was filtered through membrane filters (Millipore Corp., Billerica, US) of size 0.45 µm, which were diluted with triple distilled water and analyzed by HPLC assay.
**In vitro drug release study**

*In vitro* drug release studies of prepared solid dispersions and pure drug were performed in triplicate using dissolution apparatus (DS 8000, LABINDIA, Navi Mumbai, India) in distilled water at 37 ± 0.5°C using USP type II apparatus at 100 rpm. Powdered solid dispersions equivalent to 40 mg of OLM were added to the dissolution medium. At appropriate time intervals, 10 mL sample was withdrawn and replaced with fresh dissolution medium to maintain the sink conditions. The withdrawn samples were then filtered using membrane filter (Millipore Corp., Billerica, MA) and analyzed for drug content using HPLC. The dissolution efficiency (DE%) after 30 and 120 min was determined using trapezoidal method and was calculated as the percentage area of the rectangle divided by area of 100% dissolution at particular time.

**Nanocharacterization using different techniques**

**Particle size analysis**

Particle size of the solid dispersion was determined using particle size analyzer (Nano ZS90, Malvern Zetasizer, Malvern, UK). The sample was dissolved in triple distilled water and subjected to particle size analysis. This method also depicted the intensity distribution of the particles diameter and polydispersity index which is a measure of uniformity in size distribution.

**Differential scanning calorimetry (DSC)**

DSC analysis was performed using automatic differential scanning calorimeter (Shimadzu DSC-60A). Each sample (3 mg) was weighed and analyzed in pierced aluminium pans and analysis was carried out under nitrogen purge at a heating rate of 10°C/min and temperature range of 50 to 300°C.

**X-ray diffraction**

X-ray diffraction patterns can be employed for confirming the crystalline nature of the drug. Powder X-ray diffractometer (D8-Focus, Bruker, USA) was employed to carry out XRD of drug, polymers and solid dispersions using CuKα radiation.

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<th>Polymer</th>
<th>Formulation code</th>
<th>Method used</th>
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<th>DE120</th>
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<td>83.48</td>
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Table 3. Dissolution efficiency of olmesartan medoxomil solid dispersions.
Table 4. Composition and characterization of drug free tablet.

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<th>Ingredients (mg)</th>
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<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>F8</th>
<th>F9</th>
<th>F10</th>
<th>F11</th>
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<th>F13</th>
<th>F14</th>
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<tr>
<td>Hardness (kg/cm²)</td>
<td>3.2 ± 0.096</td>
<td>2.0 ± 0.070</td>
<td>3.4 ± 0.103</td>
<td>3.2 ± 0.102</td>
<td>3.3 ± 0.112</td>
<td>3.2 ± 0.117</td>
<td>3.3 ± 0.129</td>
<td>3.6 ± 0.102</td>
<td>3.2 ± 0.115</td>
<td>3.3 ± 0.121</td>
<td>3.4 ± 0.102</td>
<td>3.1 ± 0.124</td>
<td>2.8 ± 0.056</td>
<td>2.9 ± 0.087</td>
<td>2.8 ± 0.140</td>
<td>2.8 ± 0.084</td>
</tr>
<tr>
<td>Friability (%)</td>
<td>0.614 ± 0.012</td>
<td>0.725 ± 0.038</td>
<td>0.663 ± 0.033</td>
<td>0.687 ± 0.027</td>
<td>0.619 ± 0.030</td>
<td>0.607 ± 0.043</td>
<td>0.570 ± 0.059</td>
<td>0.673 ± 0.027</td>
<td>0.943 ± 0.032</td>
<td>1.279 ± 0.051</td>
<td>1.602 ± 0.024</td>
<td>1.916 ± 0.053</td>
<td>0.842 ± 0.041</td>
<td>1.257 ± 0.040</td>
<td>1.678 ± 0.035</td>
<td>1.874 ± 0.041</td>
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<td>Disintegration time (sec)</td>
<td>9 ± 1.48</td>
<td>83 ± 1.69</td>
<td>60 ± 1.15</td>
<td>39 ± 0.81</td>
<td>72 ± 1.91</td>
<td>68 ± 1.63</td>
<td>41 ± 1.48</td>
<td>32 ± 1.12</td>
<td>76 ± 1.86</td>
<td>52 ± 1.02</td>
<td>37 ± 1.08</td>
<td>23 ± 0.43</td>
<td>111 ± 2.14</td>
<td>72 ± 1.38</td>
<td>58 ± 1.73</td>
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<td>Wetting time (sec)</td>
<td>84 ± 1.69</td>
<td>67 ± 1.37</td>
<td>51 ± 2.48</td>
<td>32 ± 1.63</td>
<td>61 ± 3.26</td>
<td>53 ± 1.83</td>
<td>34 ± 2.41</td>
<td>21 ± 2.06</td>
<td>67 ± 1.48</td>
<td>41 ± 3.48</td>
<td>28 ± 2.30</td>
<td>14 ± 1.69</td>
<td>94 ± 2.08</td>
<td>61 ± 2.16</td>
<td>45 ± 3.27</td>
<td>32 ± 1.30</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± S.D. (n = 3).
in the angle 20 range from 5 to 80 with step size of 0.02, step time of 18.7 min and scanning speed of 4/min.

**Scanning electron microscopy (SEM)**

The morphology of pure drug, carrier and prepared solid dispersions was determined using scanning electron microscope (ZEISS SUPRA-55, Germany) operated at an accelerating voltage of 10 kV and aperture was 20 µm. Samples were prepared by mounting powder on a brass stub using graphite glue and coated with gold and then viewed under SEM.

**Transmission electron microscopy (TEM)**

It was carried out in order to determine the morphology (particle-shape and particle-size) of the solid dispersions. When dispersed in water, solid dispersion either forms molecular dispersion or nano-dispersion with reduced particle size. The solid dispersion was dissolved in triple distilled water and a drop was coated on a carbon coated copper grid of 200 mesh size and dried prior to the observation under transmission electron microscope, (HRTEM, JEOL, JEM 2100, Japan) operated at an accelerating voltage of 200 kV with beam current of 100 µA (29).

**Preparation of blank tablets for optimization of different components**

The drug free tablets were prepared in order to optimize the type and concentration of sublimating agent and superdisintegrant. All the raw materials were passed through a screen (44 mesh). Camphor, crospovidone, microcrystalline cellulose, mannitol and lactopress were mixed using a glass mortar and pestle. The blends were lubricated with 1% w/w of talc and 2% w/w of magnesium stearate, which were then compressed in an eight station tablet punching machine (Lab-8, Saimach, Ahmedabad, India) equipped with flat face 8 mm punches. The tablet weight was adjusted to 300 mg. The tablets were dried for 6 h under vacuum at 50°C to render the tablets porous by sublimation of sublimating agent.

**Experimental design of fast dissolving tablets**

A 3² randomized full factorial design (Design Expert version 10, State Ease Inc., USA) was used

![Figure 3. Dissolution profile of solid dispersions prepared with poloxamer 188 using A) modified solvent evaporation method, B) lyophilization; and solid dispersions prepared with modified locust bean gum using C) modified solvent evaporation method, D) lyophilization](image-url)
in order to investigate the dual influence of two formulation variables. In this design, 2 factors were evaluated, each at 3 levels, and experimental trials were performed at all 9 possible combinations. The amounts of subliming agent, camphor (X1), and the superdisintegrant, crospovidone (X2), were selected as independent variables. The disintegration time and percentage friability were selected as dependent variables. Checkpoint batch was also prepared to prove the validity of the evolved mathematical model. In addition, contour plots were used to graphically represent the effect of the independent variables.

Evaluation of tablet properties

The strength of the prepared tablets was determined using hardness tester (Monsanto) and the friability was assessed using Roche friabilator (FT1020, LABINDIA, Navi Mumbai, India). For determining friability, 20 pre-weighed tablets were taken and rotated at 25 rpm for 4 min in friabilator and then reweighed after removal of fines. From this, percentage reduction in weight indicating friability was calculated. For determining wetting time, 5 round pieces of tissue paper (0.45 µm pore size, Hi-media) were placed in a Petri dish. Appropriate quantity of water containing a watersoluble dye (eosin 0.01%), was placed on the tissue paper. A tablet was kept on the surface of the tissue paper and time required for water to reach the upper surface of the tablet was determined. For disintegration time, a modified method was used, in which 6 mL of Sorenson’s buffer (pH 6.8) at 37 ± 0.5°C was placed in a 10 mL cylindrical glass vessel, so that 2 mL of the media remained below the sieve and 4 mL above. Single tablet was placed on the sieve and entire assembly was mounted on a high precision water bath shaker (Narang Scientific Works, New Delhi, India). The time taken for all the drug particles to pass through the sieve was noted which was considered as disintegration time. Six tablets were selected randomly from each batch and were tested for disintegration time and the mean value was calculated (30-32).

RESULTS AND DISCUSSION

The choice of a particular method/carrier in the preparation of solid dispersion significantly affects
the pharmaceutical behavior of the prepared product. Owing to this, in the present study, solid dispersions were prepared using different methods in merger with different carriers in order to determine the effect of individual method or carrier on the final formulation. The locust bean gum used as carrier was modified in order to reduce its viscosity without significantly altering other parameters and the results are shown in Table 1. The viscosity was determined at different shear rate and the viscosity profile of locust bean gum and modified locust bean gum at a shear rate of 20 s$^{-1}$ and 40 s$^{-1}$ is shown in Figure 1. There is a prominent difference in viscosity with a value of 1251 ± 13 and 137.2 ± 1.5 cps at shear rate of 40 s$^{-1}$ for locust bean gum and modified locust bean gum, respectively, which showed 9-fold decrease in viscosity of the carrier on modification.

Solid dispersions were prepared in different drug to carrier ratios ranging from 1 : 1 to 1 : 5 using modified locust bean gum (natural) and poloxamer 188 (synthetic) as carriers. Similarly, two different methods viz. modified solvent evaporation and lyophilization were also evaluated for the preparation of solid dispersions. The composition of all the prepared solid dispersions of OLM prepared using both methods in unification with both carriers are given in Table 2.

Drug content of all the formulations was found in the range 94.8 ± 1.4% to 99.5 ± 0.8%. To determine the effectiveness of prepared solid dispersions, all formulations were subjected to solubility testing and the results are shown in Table 2. It was observed that as the drug to carrier ratio was increased, the solubility of the drug also increased in a linear manner with no significant difference observed after 1 : 4. So, the drug to carrier ratio of 1 : 4 was optimized in all cases. It was also found that the solubility of drug increased to higher value using lyophilization technique as compared to modified solvent evaporation which is due to the formation of porous and fluffy product by lyophilization technique which increases the surface area and, in turn, the surface free energy, resulting in higher solubility and dissolution (26). The lower is the particle size of solid dispersion; higher is the solubility which is also proved here in this present study. Among all the solid dispersions at drug to polymer ratio of 1 : 4, particle size of two solid dispersions (OLM 9 and OLM 19) prepared using lyophilization technique showed greater variability with elevated solubility as compared to the dispersions prepared with modified solvent evaporation method as shown in Table 2. The particle size of these two optimized solid dispersions was found to be 514.3 ± 2.5 nm and 58.2 ± 1.8 nm when prepared with poloxamer 188 (OLM 9) and modified locust bean gum (OLM19), respectively, as shown in Figure 2. Whereas, it was found to be 877.3 ± 4.1 nm and 238.6 ± 3.1 nm for poloxamer 188 and modified locust bean gum, respectively, for solid dispersion prepared using modified solvent evaporation method.

The results of particle size and solubility analysis proved the supremacy of lyophilization method over modified solvent evaporation method. Even the results showed a great difference in the solubility and particle size of solid dispersions prepared with both carriers also. However, this fact of carrier effect was further determined by subjecting all formulations prepared with both techniques to dissolution and nanocharacterization studies to strengthen the findings.

**In vitro dissolution studies**

The result of dissolution studies of prepared solid dispersions of OLM are shown in Figure 3. The percentage release was found to be better in case of solid dispersions prepared by lyophilization technique as compared to modified solvent evaporation.
Effect of polymer and method on particle size and crystallinity of... 1553

...tion method, whereas, it was highest when modified locust bean gum was used as carrier. In lieu to this, solid dispersions prepared using poloxamer 188 and modified locust bean gum with lyophilization technique showed dissolution efficiency (DE120) of 70.59% and 84.39% at 120 min, indicating higher dissolution efficiency using modified locust bean gum as shown in Table 3. This enhanced dissolution effect with modified locust bean gum is due to its swelling nature which results in increased surface area during dissolution resulting in higher dissolution rate of the drug (25). Moreover, modified locust bean gum is a natural polymer and the use of natural carrier for the preparation of solid dispersions is more beneficial because of their low cost, biocompatibility, and biodegradability. These findings with both carriers were further supported by carrying out various characterizations of two optimized solid dispersions showing enhanced solubility, dissolution efficiency and reduced particle size.

### Nanocharacterization of solid dispersion

Figure 4 shows FTIR spectrum of OLM, poloxamer 188, modified locust bean gum and solid dispersions prepared using both carriers. FTIR spectra of pure OLM shows characteristic peaks at 3284.90 cm⁻¹ (broad intermolecular hydrogen bond, O-H stretch), 2965.68 cm⁻¹ (aliphatic C-H stretch), 1706.01 cm⁻¹ (C=O of carboxylic group), 1476.52 cm⁻¹ (C-N stretch), 1394.67 cm⁻¹ (in plane O-H bend) and 1051.17 cm⁻¹ (ring C-O-C stretch). The solid dispersions prepared using both carriers exhibit characteristic bands of the drug and do not exhibit disappearance of any peak. This indicates nonexistence of any interaction between the drug and the carriers used.

### Determination of amorphization with DSC and XRD

DSC curves of pure drug, poloxamer 188 and solid dispersion prepared with poloxamer 188 are shown in Figure 5. For pure OLM, a sharp endothermic peak is observed at 183.05°C, characterizing melting point of OLM which indicates that the pure drug was in crystalline form. Poloxamer 188 showed a melting endothermic peak at 58.71°C. Upon formation of solid dispersion of drug with poloxamer 188, the melting endotherm of drug disappeared. On the other hand, no sharp endothermic peak was observed in the DSC curve for solid dispersion prepared with modified locust bean gum as shown in Figure 6, while it showed broadening and slight shifting of OLM peak indicating reduction in crystallinity of drug or dispersibility of drug in this
carrier. In DSC analysis of both solid dispersions, there was disappearance of the drug melting endotherm in the solid dispersion prepared with poloxamer 188, which could be due to the presence of the amorphous form of OLM in the solid dispersion. But the sharp peak corresponding to carrier remained and was at slightly lower temperature than that of pure poloxamer 188. It might be due to the reason that drug molecules get dispersed in the poloxamer 188 matrix of the solid dispersion and the thermal property was changed or it might be due to the formation of eutectic mixtures in solid dispersions leading to the depression of melting point. As in both solid dispersions drug to carrier ratios were the same, but there was incongruity in these results, so in order to deep root these findings, XRD of pure drug and its solid dispersions was carried out (33, 34).

XRD patterns of pure drug, carriers and solid dispersions are shown in Figure 7. The XRD of pure OLM showed characteristic peaks with high intensity at various 20 values which were intense and sharp, indicating its crystalline nature. These high intensity peaks diminished in case of solid dispersions prepared using poloxamer 188 whereas these characteristic peaks disappeared in case of solid dispersions prepared using modified locust bean gum indicating reduction in crystallinity of drug via formation of amorphous solid dispersion. The degree of crystallinity was decreased to maximum extent in case of solid dispersion prepared using modified locust bean gum by lyophilization technique. Both the above discussed parameters indicate amorphization of OLM by the formation of solid dispersions which is responsible for substantial enhancement in solubility.

Scanning electron microscopy

The photomicrographs of OLM, solid dispersions prepared with modified locust bean gum and poloxamer 188 are shown in Figure 8. Pure drug appeared as crystals whereas both carriers and solid dispersions revealed amorphous particles. There was reduction in crystallinity of drug on formation of solid dispersion; moreover, solid dispersions prepared with modified locust bean gum showed reduced particle size in nanometric range as indicated in Figure 8D, which were clearly visible only at higher magnification. The SEM studies further con-
Figure 6. DSC of olmesartan medoxomil pure drug, modified locust bean gum and solid dispersion prepared with modified locust bean gum using lyophilization method.

Figure 7. XRD of olmesartan medoxomil pure drug, poloxamer 188, modified locust bean gum and solid dispersion prepared with poloxamer 188 and modified locust bean gum using lyophilization method.
firmed the result of particle size analysis, dissolution studies and XRD.

**Transmission electron microscopy**

It was carried out in order to determine the morphology (particle shape and particle size) of the solid dispersions. The particle size of solid dispersion prepared with modified locust bean gum was found to be 36.59 nm whereas that prepared with poloxamer 188 was 505.68 nm as shown in Figure 9, indicating smallest particle size and higher solubility with modified locust bean gum. Also, solid dispersion prepared with modified locust bean gum revealed regular spherical shape, due to which it has shown acceptable flow properties. The reduced particle size is responsible for its improved solubility and dissolution rate as compared to solid dispersion prepared with poloxamer 188 and pure OLM.

Besides cost effective, natural, biocompatible and biodegradable polymer, modified locust bean has also proved to be a potential carrier for improving the solubility and reducing particle size of OLM. It revealed better results as compared to poloxamer 188. Similarly, lyophilization method has proved to be the most appropriate method for preparation of solid dispersions. Based on these results, OLM19 prepared with lyophilization technique along with modified locust bean gum as carrier showing highest solubility, reduced particle size, spherical and amorphous particles and reduced crystallinity was optimized for preparation of fast dissolving tablets.

**Preparation of drug free tablets**

The compositions of drug free tablets are shown in Table 4. Different superdisintegrants and sublimating agents were used in order to determine

---

**Table 7. Regression analysis data of olmesartan medoxomil.**

<table>
<thead>
<tr>
<th>Response</th>
<th>Disintegration time (sec)</th>
<th>Friability (%)</th>
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<tr>
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<td>FM</td>
<td>RM</td>
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<td>49.66667</td>
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<tr>
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<tr>
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<td>$b_{22}$</td>
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<td>-</td>
</tr>
<tr>
<td>$b_{12}$</td>
<td>-2.75000</td>
<td>-</td>
</tr>
</tbody>
</table>

* FM = Full model, RM = Reduced model

**Table 8. Results of ANOVA of full models and reduced model for dependent variables.**

<table>
<thead>
<tr>
<th>For disintegration time</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
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<th>$R^2$</th>
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<td>3</td>
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<td>2734.17</td>
<td>1367.08</td>
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<td></td>
<td>6</td>
<td>133.83</td>
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<td>-</td>
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</table>

<table>
<thead>
<tr>
<th>For friability</th>
<th>df</th>
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<th>MS</th>
<th>$f$</th>
<th>$R^2$</th>
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<td>-</td>
</tr>
<tr>
<td>Reduced model</td>
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<td>0.088</td>
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</tr>
<tr>
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<td>6</td>
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<td>0.00073</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

* df = degree of freedom, SS = sum of squares, MS = mean of squares, $f$ = Fischer's ratio, $R =$ Regression coefficient.
their effect to optimize better one. All prepared tablets showed uniformity in weight and hardness. Crospovidone decreased the disintegration time more effectively as compared to Ac-Di-Sol when used in equal concentration, wherein at a concentration of 3% w/w it was found to be 41 ± 1.48 and 60 ± 1.15 s for crospovidone and Ac-Di-Sol, respectively. No significant difference in disintegration time was observed after this concentration. The same was observed in the case of sublimating agent where camphor showed prominent results. But on increasing the concentration of camphor beyond 3%, friability of tablets started increasing although the disintegration time was decreased. It is stated that the superdisintegrant act through strain recovery, wicking and capillary action (35) and sublimating agent generate porosity in the tablet which increases water uptake and, in turn, facilitate the wicking action of superdisintegrant (36). Both crospovidone and camphor when used in combination showed better result.
Figure 9. TEM of solid dispersion prepared by poloxamer 188 (A) and modified locust bean gum (B)

Figure 10. Response surface plot of disintegration time and % friability (A), contour plot of disintegration time and % friability (B), response surface prediction plot (C)
as compared to those when used alone (37). So, on the basis of disintegration time, crospovidone and camphor were selected as superdisintegrant and sublimating agent to be used in combination in the concentration of 1-3% for the preparation of tablets.

Experimental design of fast dissolving tablets

A 3 factorial design was used in order to optimize the amount of both subliming agent and superdisintegrant to obtain the desired disintegration time and friability. The amount of camphor ($X_1$) and crospovidone ($X_2$) were chosen as independent variables. A statistical model incorporating both interactive and polynomial terms was used to estimate the response by using equation

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2; \quad (2)$$

$Y$ is dependent variable (disintegration time and friability), $b_0$ is arithmetic mean response of the all 9 runs, and both $b_1$ and $b_2$ are estimated coefficients for $X_1$ and $X_2$, respectively. Here $X_1$ and $X_2$ provide the average result on varying a single factor at one time, whereas $X_1X_2$ is the interaction term which illustrates how the response changes when 2 factors are changed simultaneously. Both polynomial terms i.e., $X_1X_1$ and $X_1X_2$ are included to determine nonlinearity. The composition of all prepared nine formulations is shown in Table 5.

There was a significant difference in disintegration time from $80 \pm 1.60$ to $20 \pm 1.59$ s where % friability also showed a wide variation from $0.611 \pm 0.017\%$ to $0.443 \pm 0.014\%$ in all the prepared batches. It is clearly depicted from Table 6 that both the chosen independent variables have significant effect on disintegration time and % friability. The fitted equations (full and reduced models) relating different responses, disintegration time, and % friability to the transformed factor are revealed in Table 7.

The polynomial equations can be utilized to draw conclusions from the magnitude of coefficient and positive or negative sign. The results of the analysis of variance (ANOVA) as shown in Table 8, were executed to identify the insignificant factors. The value of correlation coefficient was near to 1 for both disintegration time or percentage friability, thereby indicating a good fit for all dependent variables. Among both independent variables (disintegration time and friability), regression analysis indicate that coefficients $b_{11}$, $b_{22}$, $b_{12}$ ($p \leq 0.05$) were insignificant in predicting disintegration time and friability. Hence, these terms were omitted from full model to generate reduced model.

Both coefficients $b_1$ and $b_2$ bear a negative sign as shown in multiple linear regression analysis (reduced model) which indicate that on increasing the concentration of either camphor or crospovidone, the disintegration time decreases. This is due to the fact that higher percentage of camphor induces higher porosity facilitating higher water uptake which leads to reduced disintegration time. On the contrary, this increase in the concentration of camphor increased friability as the coefficient $b_1$ bears a positive sign. However, on the other hand, negative sign of the coefficient $b_2$ indicated that on increasing the concentration of crospovidone, the friability decreased and mechanically strong tablets were produced.
Optimization of formulation variables

The optimization of tablet components (camphor and crospovidone) was undergone to target the disintegration time and % friability of 30 s and 0.6% respectively. The optimized amount determined with the help of software is depicted in surface response curves as shown in Figure 10.

A checkpoint batch (OCP) was prepared at $X_1 = 0.99$ level, and $X_2 = 0.18$ level at which disintegration time and % friability was 31.99 s and 0.599%, respectively. The desirability of the optimized batch was 0.980 which is almost near to 1. From the reduced model, it was found that the disintegration time of the checkpoint batch (OCP) was $31 \pm 1.06$ s and friability was $0.602 \pm 0.013\%$. The optimized batch (OCP) depicted the expected results, therefore this model is statistically valid. It was found that the observed responses of the prepared optimized preparation were similar to the predicted values indicating the feasibility of this optimization process. The optimized batch producing fast dissolving tablets was prepared and compared with commercial tablet and the results are shown in Figure 11. The prepared tablets revealed fast and improved dissolution as compared to commercial tablet within a time period of only 5 min which indicates better effect of prepared formulation for immediate treatment of hypertension.

CONCLUSION

Highly soluble solid dispersions of OLM with enhanced efficacy were resulted from the use of a natural carrier (modified locust bean gum), bypassing the utilization of any surfactant or solubilizing agent. This optimized solid dispersion prepared with modified locust bean gum in union with lyophilization technique showed the smallest particle size of 58.8 ± 1.8 nm and higher solubility of 1097 ± 15.92 µg/mL. This is due to the fact that lyophilization method results in porous and fluffy product which increases the surface area and in turn the surface free energy, resulting in higher solubility; while modified locust bean gum possess swelling nature which also results in increased extensive surface area during dissolution leading to higher dissolution rate of the drug. It was found that the particle size and solubility of the dispersion is attributed to the type of carrier and method used. The solid dispersion prepared with lyophilization technique along with modified locust bean gum as carrier showing superior properties was optimized and was formulated into fast dissolving tablet to attain quick action which can be used as a successful alternative for already available marketed tablets of OLM to triumph over its drawback of low solubility and low bioavailability.

Conflict of interest

The authors confirm that this article content has no conflicts.

REFERENCES


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FORMULATION AND COMPATIBILITY ASSESSMENT OF PLGA/LECITHIN BASED LIPID-POLYMER HYBRID NANOPARTICLES CONTAINING DOXORUBICIN

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¹Department of Pharmacy, The Islamia University Bahawalpur, Pakistan
¹Institute of Pharmacy, Physiology and Pharmacology, University of Agriculture, Faisalabad, Pakistan

Abstract: In the world of materials, many individual components don’t meet the need of drug delivery system for optimum therapeutic outcomes. However, the combination of materials provides this opportunity. The success of such combinatorial system is based on the compatibility of different formulation components with each other. The presence of any physical or chemical interaction affect the outcomes of the system in terms of stability, bioavailability, and entrapment of the active ingredient. ATR-FTIR spectrometry have been extensively employed in the investigation of compatibility among the different pharmaceutical ingredients. The present study was planned to study any possible interaction among the doxorubicin, PLGA and lecithin in resulting physical mixtures and lipid polymer hybrid nanoparticles. All physical mixtures were prepared in equimolar ratios by simple blending, while nanoparticles with modified single step nanoprecipitation method combined with self-assembly. The particle size range from 178 to 197 nm with polydispersity index (PDI) of 0.14 to 0.25, while the zeta potential varied from -17 to -24.5 mV. The spectra of doxorubicin indicate the characteristic peaks at 3320 cm⁻¹ (N-H stretching), 1730 cm⁻¹ (carbonyl group) and at 1615 cm⁻¹ and 1580 cm⁻¹ for amide I and amide II bands. The IR spectra of PLGA indicate the ester carbonyl group symmetric stretching (–C=O) at 1747 cm⁻¹, deformational vibration of –C-H group at 1452 cm⁻¹ and terminal hydroxyl group at 3501 cm⁻¹; while lecithin at 2922 cm⁻¹ (stretching vibration of methylene group), 1229 cm⁻¹ (PO₂), and 1065 cm⁻¹ (P-O-C stretching). The slight shift of the peaks of doxorubicin at 3270 cm⁻¹ (N-H stretching) indicate the successful encapsulation of drug in nanoparticulate formulations. The results suggested no significant interaction of doxorubicin with the PLGA and lecithin, when combined in the physical mixtures and the lipid polymer hybrid nanoparticles.

Keywords: compatibility studies, doxorubicin, ATR-FTIR, lipid-polymer hybrid nanoparticles, physical mixture

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Increasing incidence of cancer is a global dilemma involving multiple environmental and physiological factors. The chemotherapy is an effective approach to treat different types of cancer, such as breast cancers, and prostate cancers (1, 2). However, lack of targetability and specificity to the tumor cell, the emergence of multiple side effects and multidrug resistance, toxicities and uncontrolled therapeutic levels hindered therapeutic success. Doxorubicin hydrochloride (DOX) is a chemotherapeutic agent that is classified as class I non-selective anthracycline antibiotics that were firstly obtained from Streptomyces peucetius (3). Chemically, DOX is (7S, 9S)-9-hydroxyacetly-4-methoxy-7,8,9,10-tetrahydro-6,7,9,11-tetrahydroxy-7-O-(2’3’6’-trideoxy-3’-amino-α-L-lyxohexopyranosyl)-5,12-naphthaceninedione, as represented in Figure 1. It consists of a sugar group (daunosamine) attached to an aglycone component, comprising of a tetracyclic ring with quinine-hydroquinone functional groups. Methoxy substituent is attached to a carbonyl group that provides the site for further interaction with other components (4).

Mechanistically, DOX affects the DNA replication process by interacting with the enzymes topoisomerase I and II that are responsible for the uncoiling of double stranded DNA. The hindrance in the replication process results in programmed cell
death (5, 6). It is recommended in different malignancies and carcinomas such as solid tumors, lymphomas, leukemia, breast and lung carcinomas by the Food and Drug Administration, USA (7, 8).

In the past few decades, nanotechnology has been considered a forefront area of research to develop multifunctional nano-carriers for the delivery of anti-cancer drugs. The present study has been designed to formulate these nano-carriers from different natural and synthetic polymers to get sustained, targeted, and delivery of an effective concentration of chemotherapeutic (9, 10). Combining of polymeric nanoparticles and liposomal drug carriers as lipid-polymer hybrid nanoparticles have offered distinct advantages of improved solubility, controlled release, enhanced residence time due to less opsonization and targeted delivery with fewer side effects and enhanced patient compliance. (11). These systems have merged the advantages of both individual carrier systems and addressed their shortcomings (12). Other advantages of this system are the biocompatibility, biodegradability and high encapsulation of therapeutic moieties while the coated lipid potentially acts as a barrier to prevent the drug leakage and degradation of the inner polymeric core (13).

In the process of drug design and preformulation studies, any chemical and/or physical incompatibility is considered a major hindrance for obtaining the desired therapeutic objectives. Therefore, the compatibility analysis of the active pharmaceutical ingredient (API) and other formulation excipients is a determinant factor in the pre and post formulation investigations (14). Most of the excipients lack direct pharmacological effects but it may involve in some inadvertent processes such as polymer degradation and formation of newer compounds. Thus, the chemical nature, formulation stability, and bioavailability of the API is directly influenced by the excipients (15, 16). A limited data are available for the compatibility studies of hybrid nanoparticle systems. The studies have been conducted by differential scanning calorimetry (DSC), thermogravimetry (TG), and isothermal titration calorimetry whereas, the non-thermal compatibility studies involve different spectroscopic methods, including Fourier transform infrared (FTIR) spectroscopy, diffuse reflectance spectroscopy (DRS), X-rays diffraction (XRD) and nuclear magnetic resonance (NMR) (17-19).

In the present study, attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy has been employed as a reliable tool for the prompt identification of different API, excipients and their physical or chemical interaction based on the shifting of the bands due to presence of bonding

![Doxorubicin](image)

**Figure 1. Structure of doxorubicin**

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Equivalent ratios</th>
<th>Chemical description</th>
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<tbody>
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<td>A</td>
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<td>Doxorubicin</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>PLGA 50 : 50</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>PLGA 75 : 25</td>
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<tr>
<td>D</td>
<td>1</td>
<td>Lecithin</td>
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<tr>
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<td>1 : 1</td>
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<td>PM7</td>
<td>1 : 1 : 1</td>
<td>Doxorubicin + PLGA 75 : 25 + Lecithin</td>
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</table>
between functional groups (20). ATR-FTIR technique can be employed for qualitative and quantitative analysis of samples and is therefore considered as an important technique to find out the interaction or incompatibility by producing the unique peaks or band shifted from their normal positions (21). The sample of liquid, solid or gas is analyzed by the attenuated total reflection mode and is reflected by a broadening of existing peaks, appearance of new peaks and overlapping with higher intensities are the fundamental evidence to explain the possible interactions (22, 23).

Therefore, the present study has been undertaken to develop lipid-polymer hybrid nanoparticles, binary and tertiary physical mixtures of different structural components. The compatibility studies between doxorubicin and lecithin, and/or poly lactic-co-glycolic acid (PLGA). Drug-lipid and drug-polymer interactions were analyzed in the formulations and all physical mixtures by using ATR-FTIR spectroscopy technique.

### EXPERIMENTAL

#### Materials

Doxorubicin (C_{27}H_{29}NO_{11}, PubChem CID: 31703) was purchased from Beijing Mesochem Technology Co., Ltd. (China). PLGA (poly (D, L-lactic-co-glycolic acid, 75 : 25 and 50 : 50) ([C_3H_4O_2]x[C_2H_2O_2]_y, PubChem CID: 23111554) RESOMER® was purchased from Evonik Chemicals, Lecithin (C_{35}H_{66}NO_7P, PubChem CID: 57369748) from Xi’an Rongsheng Biotechnology Co. Ltd. (China) and all other reagents and solvents used in the present study were of analytical grade.

#### Preparation of physical mixtures

The physical mixtures of drug, polymer and lipid were prepared by taking equimolar amounts (1 : 1 w/w) through physical mixing approach. All the components were mixed in mortar with pestle for 15 min to ensure the uniform mixing and obtaining a

<table>
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</thead>
<tbody>
<tr>
<td>F₁</td>
<td>-</td>
<td>60</td>
<td>-</td>
<td>09</td>
</tr>
<tr>
<td>F₂</td>
<td>10</td>
<td>60</td>
<td>-</td>
<td>09</td>
</tr>
<tr>
<td>F₃</td>
<td>10</td>
<td>-</td>
<td>60</td>
<td>09</td>
</tr>
</tbody>
</table>

Table 2. Composition of blank and drug-loaded LPHNPs.
uniform blend (24). The physical mixtures combinations were coded and analyzed as given in Table 1.

Fabrication of lipid-polymer hybrid nanoparticles

A single step modified nanoprecipitation method in combination with the self-assembly was employed to fabricate the lipid-polymer hybrid nanoparticles (LPHNPs) of PLGA (poly (D, L-lactic-co-glycolic acid) and lecithin, as described by Zhang et al. (25). A suitable amount of PLGA was dissolved in acetonitrile to form 2 mg/mL and 3 mg/mL solutions. Lecithin (15%) of the total polymer weight was added to the 4% hydroalcoholic solution and heated at 65°C to form a uniform solution. Then, the polymer solution was added at a rate of 1 mL/min into the preheated lecithin solution with constant stirring at 65°C and vigorously vortexed for 5 min and subjected to gentle stirring at 850 rpm for 2 h for the self-assembling of the HNPs. The organic solvent was evaporated and HNPs were collected.

DOX-loaded HNPs were prepared by the same method. The drug (doxorubicin) was added into 4% ethanolic aqueous solution along with the lecithin in the properly calculated dose before mixing with the organic phase as given in Table 2. The collected HNPs were washed three times using ultracentrifuge Sigma 1-14 (Sigma Laborzentrifugen GmbH, Germany) to remove the unentrapped drug and lyophilized (CHRIST Alpha 1-2 LD plus, UK) for 24 h.

Characterization of the LPHNPs

Determination of particle size, size distribution and zeta potential

The particle size and the particle size distribution (polydispersity index) of blanks and DOX loaded LPHNPs were analyzed by the dynamic light scattering (DLS). Whereas, the surface charge (zeta potential) was measured on the electrophoretic mobility basis by using the Malvern zeta Sizer Nano ZS instrument (Malvern Instruments Ltd, UK). The samples 25 µL of nanoparticle were diluted with the 975 µL with distilled water prior to analysis.

Surface morphology of the LPHNPs

The morphology of the nanoparticles was analyzed by using the scanning electron microscope (SEM) (Quanta 250 FEG, FEI, America). This analysis was performed by sprinkling tests through sticky tape stuck on aluminum stub and all the photomicrographs were recorded at 5 kV with different amplification powers.

Attenuated total reflectance infrared Fourier transform spectroscopy (ATR-FTIR)

ATR-FTIR spectroscopy was employed to identify the physical or chemical interaction between the drug and other formulation components. The FTIR spectra of doxorubicin, polymer or lipid alone, binary mixtures of the drug with polymer and lipid, tertiary blends of drug, lipid, and polymer and the lipid-polymer hybrid nanoparticle formulations (blank and drug-loaded) were obtained by using Bruker tensor 27 series (Germany) FTIR spectrometer (26). The instrument utilized pike single bounced attenuated total reflectance method containing Zinc-Selenium (ZnSe) single crystal standard sample cell. The samples were placed at the interface of direct infrared light and internal reflection element (ZnSe) (27). All the spectra were obtained by scanning the sample over a range of 4000-400 cm⁻¹ with 16 scans per sample at an average resolution of 4 cm⁻¹.

RESULTS AND DISCUSSION

Particle size, size distribution and zeta potential

The particle size was found to be in the size range of 178 to 197 nm with particle size distribution (PDI) from 0.14 to 0.25, whereas, zeta potential varies from -17 to -24.5 mV (Table 3). The blank nanoparticles were smaller in size (178 nm) compared to the DOX-loaded nanoparticles (F1 and F2). The amount of drug encapsulated in the PLGA core might be responsible for this increase in size, which is in accordance with many previous studies (28, 31-33).

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Particle size* (nm)</th>
<th>Polydispersity index (PDI)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_b$</td>
<td>178 ± 0.9</td>
<td>0.14 ± 0.01</td>
<td>-24.5 ± 0.90</td>
</tr>
<tr>
<td>$F_1$</td>
<td>197 ± 3.0</td>
<td>0.22 ± 0.019</td>
<td>-17.1 ± 2.20</td>
</tr>
<tr>
<td>$F_2$</td>
<td>190 ± 2.3</td>
<td>0.25 ± 0.031</td>
<td>-17.3 ± 0.35</td>
</tr>
</tbody>
</table>

*All the results are triplicate and represented with ± SD (standard deviation).
Surface morphology of the LPHNPs

Scanning electron microscopy was employed for the morphological analysis (shape and surface) of the nanoparticles. The nanoparticles were generally spherical shape particles that confirmed the successful formulation of LPHNPs as shown in Figure 2. The irregular shaped particles and micron size particles are the self-assembled lipid particles that remain after the centrifugation in the formulation dispersion. However, for the analysis of distinct layers of the polymeric core and lipid shell, high resolution transmission electron microscopy (HR-TEM) might be required.

FT-IR spectroscopic analysis

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) is successfully employed in the pharmaceutical, chemical and biotechnological industries for compatibility studies of active and/or inert ingredients of various formulations (27, 29). The FTIR spectra of individual com-
ponents, binary and tertiary physical blends and lipid-polymer hybrid nanoparticle formulations were obtained and depicted for any possible interaction.

FT-IR spectra of individual ingredients

The FTIR spectra bearing the characteristic peaks of doxorubicin, PLGA 75 : 25, PLGA 50 : 50 and lecithin have been shown in Figure 3. The important bands and their respective functional groups of all these entities have also been summarized in Table 4. FTIR fingerprints of doxorubicin in Figure 3 (a), indicated the primary band of N-H asymmetric stretching at 3320 cm⁻¹ and peak at 2935 cm⁻¹ specifying the C-H stretching vibration (30).

Table 4. Functional groups and characteristic peaks of individual ingredients.

<table>
<thead>
<tr>
<th>Individual components</th>
<th>Functional groups</th>
<th>Characteristic peaks</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>N-H, C-H, C=O, N-H (amide I, II), C-C, C-O-C, &amp; C-O</td>
<td>3320 cm⁻¹, 2935 cm⁻¹, 1730 cm⁻¹, 1615 cm⁻¹, 1580 cm⁻¹, 1413 cm⁻¹, 1211 cm⁻¹, 1071 cm⁻¹ and 1004 cm⁻¹</td>
<td>(9, 32, 33)</td>
</tr>
<tr>
<td>PLGA 75 : 25</td>
<td>-C-H, -C≡O, -C-H, -O-CH₂-, -C=O, -C-H, -C-O-C, -C-C, -CH₂-S</td>
<td>2995 cm⁻¹ and 2946 cm⁻¹, 1747 cm⁻¹, 1452 cm⁻¹, 1423 cm⁻¹, 1382 cm⁻¹, 1268 cm⁻¹, 1182 cm⁻¹, 1047 cm⁻¹, 866 cm⁻¹, 749 cm⁻¹, 709 cm⁻¹</td>
<td>(34, 35)</td>
</tr>
<tr>
<td>PLGA 50 : 50</td>
<td>Terminal -OH, -C-H, C=O, C-H, -O-CH₂-, -C-O, -C-H, -C-O-C, -C-C, -CH₂-S (thioether)</td>
<td>3501 cm⁻¹, 2947 cm⁻¹, 1748 cm⁻¹, 1452 cm⁻¹, 1423 cm⁻¹, 1383 cm⁻¹, 1270 cm⁻¹, 1165 cm⁻¹, 868 cm⁻¹, 749 cm⁻¹ and 709 cm⁻¹</td>
<td>(36, 37)</td>
</tr>
<tr>
<td>Lecithin</td>
<td>C-H, -CH₂, C=O, C-H, P=O, P-O-C, N(CH₃)₃</td>
<td>2922 cm⁻¹, 2852 cm⁻¹, 1736 cm⁻¹, 1455 cm⁻¹, 1229 cm⁻¹, 1065 cm⁻¹, 970 cm⁻¹</td>
<td>(38)</td>
</tr>
</tbody>
</table>

Table 5. Functional groups and characteristic peaks of physical mixtures.

<table>
<thead>
<tr>
<th>Physical mixtures</th>
<th>Functional groups</th>
<th>Characteristic peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM1</td>
<td>N-H, C-H, C=O, N-H (amide I, II), C-H, P=O, P-O-C, N'(CH₃)₃</td>
<td>3317 cm⁻¹, 2925 cm⁻¹, 1730 cm⁻¹, 1616 cm⁻¹ and 1580 cm⁻¹, 1284 cm⁻¹, 1235 cm⁻¹, 1072 cm⁻¹ and 969 cm⁻¹</td>
</tr>
<tr>
<td>PM2</td>
<td>-OH, N-H, C=O, N-H, -C-O, C-O-C, C-C, CH₂-S</td>
<td>3526 cm⁻¹, 3322 cm⁻¹, 1730 cm⁻¹, 1615 cm⁻¹, 1376 cm⁻¹, 1072 cm⁻¹, 1004 cm⁻¹, 869 cm⁻¹, 709 cm⁻¹</td>
</tr>
<tr>
<td>PM3</td>
<td>N-H, C=O, C-C, C-H, C-O-C, -C-O</td>
<td>3323 cm⁻¹, 1730 cm⁻¹, 1413 cm⁻¹, 1283 cm⁻¹, 1071 cm⁻¹, 1004 cm⁻¹</td>
</tr>
<tr>
<td>PM4</td>
<td>-CH₂, C=O, C-H, P=O, P-O-C, N'(CH₃)₃</td>
<td>2852 cm⁻¹, 1737 cm⁻¹, 1454 cm⁻¹, 1230 cm⁻¹, 1066 cm⁻¹, 971 cm⁻¹</td>
</tr>
<tr>
<td>PM5</td>
<td>C=O, C-H, -C-O, P=O, P-O-C, P-O-C, N'(CH₃)₃</td>
<td>1740 cm⁻¹, 1454 cm⁻¹, 1395 cm⁻¹, 1228 cm⁻¹, 1168 cm⁻¹, 1066 cm⁻¹, 970 cm⁻¹</td>
</tr>
<tr>
<td>PM6</td>
<td>N-H, C-H, C=O, C-H, C-C, P=O, -C-O</td>
<td>3322 cm⁻¹, 2923 cm⁻¹, 1730 cm⁻¹, 1462 cm⁻¹, 1413 cm⁻¹, 1234 cm⁻¹, 1004 cm⁻¹</td>
</tr>
<tr>
<td>PM7</td>
<td>N-H, C-H, C=O, N-H, P=O, C-O-C, N'(CH₃)₃</td>
<td>3324 cm⁻¹, 2923 cm⁻¹, 1730 cm⁻¹, 1617 cm⁻¹, 1580 cm⁻¹, 1234 cm⁻¹, 1072 cm⁻¹, 870 cm⁻¹</td>
</tr>
</tbody>
</table>

Table 6. Functional groups and characteristic peaks of lipid-polymer hybrid nanoparticle formulations.

<table>
<thead>
<tr>
<th>Formulations codes</th>
<th>Functional groups</th>
<th>Characteristic peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₁</td>
<td>C-H, C=O, N-H (amide I, II), -C-O, C-O-C, P=O</td>
<td>2919 cm⁻¹, 1753 cm⁻¹, 1626 cm⁻¹, 1527 cm⁻¹, 1385 cm⁻¹, 1183 cm⁻¹, 1088 cm⁻¹</td>
</tr>
<tr>
<td>F₂</td>
<td>N-H, C=O, N-H (amide I, II), -C-O, C-C, C-O-C</td>
<td>3268 cm⁻¹, 1748 cm⁻¹, 1624 cm⁻¹, 1525 cm⁻¹, 1385 cm⁻¹, 1186 cm⁻¹, 1081 cm⁻¹</td>
</tr>
<tr>
<td>F₃</td>
<td>N-H, C=O, N-H (amide I, II), -C-O, C-C, C-O-C</td>
<td>3270 cm⁻¹, 1752 cm⁻¹, 1625 cm⁻¹, 1524 cm⁻¹, 1386 cm⁻¹, 1187 cm⁻¹, 1084 cm⁻¹</td>
</tr>
</tbody>
</table>
Figure 4. FTIR spectra of physical mixture (PM1-PM7)
The peak at 1730 cm\(^{-1}\) showed the presence of the C-O group, whereas, sharp peaks at 1615 cm\(^{-1}\) and 1580 cm\(^{-1}\) have been considered as characteristic peaks for bending vibration of (N-H) amide I and amide II groups, that overlap with the carbonyl groups of the anthracene ring (31). The peaks at 1413 cm\(^{-1}\), 1211 cm\(^{-1}\), 1071 cm\(^{-1}\) and 1004 cm\(^{-1}\) verified the C-C stretching, C-O-C asymmetric stretching vibration and C-O stretching, respectively (32).

The IR spectral analysis of polymer PLGA 50 : 50 and PLGA 75 : 25 are shown in Figure 3 (b) and (c). The band at 2995 cm\(^{-1}\) and 2946 cm\(^{-1}\) indicated the ñC-H symmetrical stretching at CH and methyl (CH\(_3\)) group. These bonds and stretching vibrations have been associated with the lactic acid monomer of the PLGA polymer. A sharp peak at 1747 cm\(^{-1}\) corresponds to the ester carbonyl group symmetric stretching (ñC=O) found at a similar position as described by the Pirooznia (34). The distinct peaks located at 1452 cm\(^{-1}\), and 1423 cm\(^{-1}\) ensure the presence of a glycolic acid fraction of the polymer. These signals revealed the deformational vibration of the bonds between carbon and hydrogen atoms (C-H) of O-CH\(_2\)-group. The signal at 1382 cm\(^{-1}\) was attributed to the saturated C-H bending in the ñCH\(_3\) (methyl) group (34, 39). The bands present at 1268 cm\(^{-1}\) disclose the C-O-C stretching while the peaks at 1182 cm\(^{-1}\) and 709 cm\(^{-1}\) corresponded to the symmetrical stretching of an ether group (C-O-C) and thioether (-CH\(_2\)-S) group, respectively. The vibrational bands at 866 cm\(^{-1}\), and 749 cm\(^{-1}\) were associated with the stretching of C-C bonds. However, in the FTIR spectra of PLGA 50 : 50 indicate a peak at 3501.78 cm\(^{-1}\) disclose the presence of terminal hydroxyl (-OH) group (36).

In the IR spectra of lecithin [Fig. 3 (d)] are the characteristic peaks of C-H (stretching vibration of methylene group) at 2922 cm\(^{-1}\) and symmetrical stretching of ñCH\(_2\) of alkyl chain at 2852 cm\(^{-1}\) (40). The stretching vibrations of the C=O group (between the hydrophilic head and lipophilic tail) exhibited at 1736 cm\(^{-1}\) and C-H deformation and scissoring vibrations were observed at wavenumber 1455 cm\(^{-1}\). The asymmetric stretch of P=O (overlapped with P-O), P-O-C stretching and N+(CH\(_3\)) stretch were depicted at 1229 cm\(^{-1}\), 1065 cm\(^{-1}\), and 970 cm\(^{-1}\), respectively (41, 42).

**FTIR spectra of physical mixture**

The physical mixtures of drug, polymer, and lipid were prepared in equimolar ratios and analyzed
for any possible physical/chemical incompatibility by using their IR spectra (Fig. 3).

The characteristic peaks of all the individual components (Fig. 3) were compared with the IR spectra of all physical mixtures (PM1-PM7) and have been summarized in Table 5. The binary mixture of doxorubicin with the lecithin (PM1) and polymer (PM2 and PM3) indicated the presence of characteristic peaks of C=O at 1730 cm\(^{-1}\) and N-H (amide I and amide II) group at 1616 cm\(^{-1}\) and 1580 cm\(^{-1}\). Similarly, the IR spectra of a tertiary mixture containing doxorubicin, lecithin and PLGA 50 : 50/PLGA 75 : 25 (PM6 and PM7) also indicated the presence of characteristic peaks of these components without any prominent shift. These results demonstrate the absence of any interaction between the drug and other formulation ingredients.

**FTIR analysis of LPHNP formulations**

The lipid-polymer hybrid nanoparticles prepared by modified nanoprecipitation method combined with the self-assembling process with different concentrations of PLGA and lipid have been presented in Table 6. The IR spectrum of blank nanoparticles (Fig. 5-F\(_b\)) indicated the intense signals of carbonyl (C=O) group with a slight shift in wave number at 1753 cm\(^{-1}\), which might be due to the overlapping of identical groups in lecithin and PLGA (43). Similarly, the characteristic peaks of the C-O-C group were identified in the region of 1047 cm\(^{-1}\) in the PLGA (Fig. 3) while the group P-O-C in lecithin indicate the stretching vibration at 1065 cm\(^{-1}\).

In hybrid nanoparticle formulations, a broader band was observed at 1081 cm\(^{-1}\) (1080-1090 cm\(^{-1}\)) resulted from the slight shifting of above-mentioned peaks indicating the presence of both the components in the formulation.

The peak at 3268 and 3270 cm\(^{-1}\) indicating the N-H stretching of doxorubicin in drug-loaded formulations (Figs. 5-F, and 4-F\(_b\)) was not observed in the IR spectra of the blank formulation (Fig. 5-F\(_b\)), which illustrated the successful entrapment of the drug without any chemical interaction. It was confirmed that the characteristic peaks pertaining to lecithin and PLGA were well conserved in the IR spectra of physical mixture and formulations with slight variation in the wave number. Considering all the data analyzed, it was suggested that no physicochemical interaction was observed in the physical mixtures and prepared LPHNPs formulations (44, 45).

**Conclusion and future prospective**

Lipid-polymer hybrid nanoparticles ranging from 178 to 197 nm were successfully fabricated with modified single step nanoprecipitation method combined with self-assembly. The non-destructive approach using ATR-FTIR spectrometry analysis confirmed the absence of physical or chemical incompatibility between doxorubicin and the respective formulation components. The absorption spectrum of doxorubicin exhibited no significant variation with PLGA, lecithin, their physical mixture, and in formulation system. A slight shift in the characteristic peaks of DOX was observed in the hybrid nanoparticulate system, which indicated the successful encapsulation in the polymer-lipid system and absence of any interaction. However, this study revealed the stable nature of doxorubicin, which predicted its possibility for the preparation of PLGA-lecithin hybrid nanoparticles. However, the compatibility and stability of such novel nano-systems will be further investigated by other techniques that might help in the clinical application of such delivery systems.

**REFERENCES**


Received: 5. 12. 2016
Therapeutic benefit of large number of chemotherapeutic drugs administered in a traditional way is sometimes limited due to poor biopharmaceutical physicochemical properties and issues of drug toxicity. Most of these chemotherapeutic drugs have poor aqueous solubility and dissolution that effect the formulation process and also limit their therapeutic benefit (1). Poor aqueous solubility not only hamper the uptake, transport and distribution but also effect bioavailability of a drug. Several techniques have been used to increase drug solubility including complexation with physically and chemically stable cyclodextrin macromolecules (2, 3). Due to superior complexing properties of HP-β-CD, it is most preferred for complexation (4, 5). These starch derivatives collaborates via dynamic complexation in a manner that hides undesired properties of multiple drugs.

5-Fluorouracil, an antimetabolite is used widely in chemotherapy regimens. It is distributed and eliminated rapidly after oral intravenous injection with half-life of 8-20 min (6). It shows poor absorption with variable bioavailability. This property makes it suitable candidate for microencapsulation. In order to improve its circulation time and achieve sustained drug release, use of biodegradable and non-biodegradable polymers and incorporation of 5-FU into particulate carrier has already been employed (7-11).

The ability of polymeric based microspheres to modify release behavior of drug is well known. Microspheres have gained great deal of attention due to their control release behavior and are very suitable to gain sequential and simultaneous delivery of anticancer agents with reduced side effects.

In order to modify release of drug, different combinations of crosslinked polymeric microcarriers are prepared for sustained delivery of 5-FU with ultimate aim to improve erratic and oral bioavailability. This study describes the use of hydroxypropyl-β-cyclodextrin to investigate the possibility of complexation with 5-FU for improving solubility, dissolution, therapeutic efficacy and bioavailability of model drug. Polymeric formulations selected on the basis of in vitro results have been further studied for biological evaluation in rabbits in order to authenticate their effectiveness up to a considerable extent.

**EXPERIMENTAL**

**HPLC analysis**

A sensitive, rapid and simple HPLC method has been validated successfully for determination of 5-FU in rabbit plasma. In vivo performance of selected drug loaded microcarriers has been conducted on the basis of HPLC results, which investigates potential of developed microspheres and microbeads.

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**PHARMACOKINETIC STUDIES OF 5-FLUOROURACIL IN RABBIT PLASMA**

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1. Faculty of Pharmacy Bahauddin Zakariya University Multan, Pakistan
2. The Women University Multan, Pakistan
3. Faculty of Pharmaceutical Sciences Government College University Faisalabad, Pakistan
4. Faculty of Pharmacy Gomal University Dera Ismail Khan, Pakistan.

**Abstract:** New polymeric complex loaded microspheres and microbeads of hydroxypropyl-β-cyclodextrin polyvinyl alcohol and sodium alginate had been proposed for (5-fluorouracil) 5-FU delivery. In-vitro and in vivo experiments indicated that drug-loaded microspheres and microbeads presented, a sustained release of 5-FU compared to the 5-FU oral drug solution and that the area under curve (AUC) was increased sufficiently with promising formulation for site-specific delivery of 5-FU to the colon with no change either in physical appearance, drug content or dissolution pattern at 40°C for six months.
Pharmacokinetic analysis

Thirty six healthy albino rabbits obtained from animal house of Faculty of Pharmacy Bahauddin Zakariya University, Multan, Pakistan of weight 2.0 to 2.5 kg were selected to investigate the effect of formulations on pharmacokinetics of 5-FU obtained as a gift sample from Roche Pharmaceuticals Karachi, Pakistan. 5-FU is a popular antineoplastic agent normally used to cure carcinomas of colon. Rabbits were divided into three groups (1, 2 and 3) at random. Standard diet was given to animals, housed in a well maintained room at 25 ± 1°C. Animals were fasted for 24 h before starting the experiment but allowed a free access to water. A drug solution of 10 mg/kg was given orally to rabbits of group 1, through a feeding tube followed by rinsing with water. In the second phase of study, 10 mg/kg drug loaded microbeads were administered to rabbits of group 2. Third phase of study deals with the administration of 10 mg/kg drug loaded microspheres to group 3 of rabbits. All animals were tagged properly and retained in the wooden boxes during sampling procedure.

Blood samples of 3 mL were taken from the jugular vein of each rabbit and collected in a citrated tubes at 10, 15, 20, 25, 30, 40, 50 min and up to 1 h from a control group1 and up to 24 h from group 2 and group 3 after successive intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h by administrating microbeads and microspheres. Plasma samples were frozen and maintained at -70°C until analysis after centrifugation. Protocols of the study were approved by Research and Ethics Committee of Faculty of Pharmacy, BZU Multan.

Pharmacokinetic profiling and quantification of plasma concentration

After administration of oral drug solution, 5-FU loaded microbeads and 5-FU loaded microspheres, plasma drug concentration of administered pure drug and drug loaded microbeads and microspheres was determined from peak area by using Microsoft Excel 2010 program.

Pharmacokinetic parameters such as maximum plasma drug concentration (Cmax), time of maximum drug concentration in plasma (Tmax), area under the plasma concentration curve (AUC), area under the first moment curve (AUMC), elimination half-life (t1/2 kel), absorption half-life (t1/2 ka), rate of absorption (Ka), rate of elimination (Ke), mean residence time (MRT), volume of distribution (Vd) and clearance (CL) were calculated using Kinetica ver 4.4.1 and are mentioned in tables 6.4 to 6.6.

Statistical analysis

Statistical analysis was performed by using one-way ANOVA in order to determine statistical significant/non-significant interpretation.

RESULTS AND DISCUSSION

HPLC analysis

In vivo performance was done to achieve separation by using (methanol : water, 20 : 80 v/v) as a mobile phase with pH adjusted at 3.2 using perchloric acid solution, maintained at a flow rate of 1mL/min. Extraction of drug was obtained by spiking 500 µL of plasma with 50 µL of drug. Perchloric acid solution (10% v/v) was then added to spiked plasma

![Figure 1. Plasma concentration vs. time (Mean ± SE) plot of 5-FU (10 mg/kg) administered oral drug solution to healthy rabbits of Group 1](image-url)
Pharmacokinetic studies of 5-fluorouracil in rabbit plasma

Sample and vortex mixed up to 10 min. Further centrifugation of sample was done at 3500 rpm for 15 min. A clear supernatant (10 µL) layer was separated and injected into HPLC system for analysis.

Pharmacokinetic analysis

Group 1
The concentrations of pure drug in plasma of 12 rabbits (group 1) after oral administration of drug are presented (Fig. 1) which illustrates (mean ± SD) of plasma drug concentration versus time. The pharmacokinetic parameters are presented in Table 1.

A constant variation in T<sub>max</sub> and C<sub>max</sub> has been reported in different studies within a similar patients. Variation of 0-80% in the bioavailability of 5-fluorouracil has been observed via oral route (12-13). Variations in pharmacokinetic parameters of 5-FU are highly dependent on dosage and route of drug administration (14). When a dose of 0.5 g was administered to eleven patients of cancer by intravenous route, only a single patient received 1.0 g paired oral and intravenous drug doses. It was observed that elimination half-life changes from 6.5 to 13.9 (min). AUC shows an increase from 807 ± 32.8 to 1537 ± 124.4 as the dose of drug increases from 0.5 g to 1 g, respectively. The values of pharmacokinetic parameters (Mean ± SD) obtained after administration of oral drug solution are comparable

![Linear regression](image)

Figure 2. Plasma concentration vs. time (Mean ± SE) plot of microbeads administered to healthy rabbits of Group 2

Table 1. Pharmacokinetic parameters of 5-FU administered as oral drug solution in rabbits of Group 1, 2 and 3.

<table>
<thead>
<tr>
<th>No.</th>
<th>Pharmacokinetics parameters</th>
<th>Drug Solution (Mean ± (SE))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>1</td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>193.069 ± 0.892</td>
</tr>
<tr>
<td>2</td>
<td>T&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>18.54 ± 0.001</td>
</tr>
<tr>
<td>3</td>
<td>AUC&lt;sub&gt;max&lt;/sub&gt; (µg.h/mL)</td>
<td>192.92 ± 0.712</td>
</tr>
<tr>
<td>4</td>
<td>AUMC&lt;sub&gt;max&lt;/sub&gt; (µg.h²/mL)</td>
<td>57.355 ± 15.911</td>
</tr>
<tr>
<td>5</td>
<td>Elimination t&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>13.2 ± 0.003</td>
</tr>
<tr>
<td>6</td>
<td>Absorption t&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>12.3 ± 0.002</td>
</tr>
<tr>
<td>7</td>
<td>K&lt;sub&gt;e&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.0561 ± 0.05</td>
</tr>
<tr>
<td>8</td>
<td>K&lt;sub&gt;a&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.513 ± 0.047</td>
</tr>
<tr>
<td>9</td>
<td>MRT (h)</td>
<td>0.499 ± 0.002</td>
</tr>
<tr>
<td>10</td>
<td>Cl&lt;sub&gt;T&lt;/sub&gt; (L/min)</td>
<td>0.0615 ± 0.002</td>
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<tr>
<td>11</td>
<td>V&lt;sub&gt;d&lt;/sub&gt; (L)</td>
<td>0.0198 ± 0.0002</td>
</tr>
</tbody>
</table>
with the previous reported studies in which similar
drug has been administered via parenteral route (14-
16). Similar work has been performed by Minhas
(17) indicating elimination half-life of oral drug
solution to be 14.607 ± 2.8509 (min), elimination
rate constant to be 0.049 ± 0.010 min−1 and values for
Cmax and AUC to be 302.387 ± 8.446 (µg/mL) and
156.536 ± 11.891 (µg.h/mL), respectively.

**Group 2**

The concentrations of 5-fluorouracil determined
in plasma of 12 rabbits (group 2) after oral adminis-
tration of formulated microbeads are presented in
Figure 2 which illustrates (mean ± SD) of plasma
concentration versus time profile of 5-FU. The phar-
macokinetic parameters are represented in Table 1.

Increase in the value of Cmax was in the agree-
ment with the reported results of Ciftci et al.
(18). The mean clearance and volume of distribution for
microbeads are 0.01335 ± 7.929 (L/min) and 0.0451
± 0.002 (L). The elimination half-life and absorption
half-life are 290.88 ± 2.220 (min) and 129.96 ±
0.021 (min). Elimination half-life was increased up
to 290.96 min, producing a sustained action in order
to maintain target concentration in body for an
extensive time period. The increase in drug half-life
is consistent with nanoparticles of 5-FU prepared
from PEG (19). Similar pattern of 5-fluorouracil
release was observed by Sastre et al. (10) where the
release of 5-FU was evaluated from poly (D,L-lact-
tide) and poly (D, L-lactide-co-glycolide) polymeric
microspheres, showed a significant increase in AUC
(up to 50 times) and MRT (up to 196 times) was
observed after injecting microspheres subcutaneously
into Wistar rats.

**Group 3**

The concentration of 5-fluorouracil determined
in the plasma of 12 rabbits (group 3) after oral
administration of microspheres are presented in
Figure 3 which illustrates (mean ± SD) of plasma
concentration versus time profile of 5-FU. The phar-
macokinetic parameters are represented in Table 1.

The mean values of Tmax and AUC were nearly
similar when compared with group 2. Similarly the
values of mean clearance and volume of distribution
are 0.0135 ± 0.0024 (L/min) and 0.0453 ± 0.0011
(L). The previous study carried out by Li et al. (19)
showed an increase in apparent volume of distribu-
tion resulting in more effective anticancer results of
5-Fu/PEG nanoparticles. The sustained release char-
acteristics of microspheres and microbeads are
reflected also in MRT of 5-fluorouracil in the body.
It has been observed that MRT considerably increas-
es after oral administration in prepared microcarriers
i.e., microbeads and microspheres up to 10.381 ±
1.829 (h) and 12.365 ± 1.310 (h) compared to oral
drug solution with a value of 0.499 ± 0.002 (h).

Desired and sustained drug release profile was
achieved by formulating microbeads and micros-
pheres which shows an increase in mean residence
time (MRT) and area under the plasma concentra-

Figure 3. Plasma concentration vs. time (Mean ± SE) Plot of 10 mg/kg) microspheres administered to rabbits of Group-3
Pharmacokinetic studies of 5-fluorouracil in rabbit plasma

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tion time curve (AUC) following microbeads and microspheres administration, when compared with oral drug solution. The same pattern of study was conducted by Zinutti et al. (20) following oral microspheres administration. Similarly increased area under the curve, 12.53 ± 1.65mg/L h vs. 7.80 ± 0.83 mg/L h and 5.82 ± 0.83 mg/L g, longer elimination half life having a value 15.43 ± 2.12 hours vs. 2.25 ± 0.22 and 1.43±0.18 hours and MRT of 7.65±0.97 hours vs. 3.61±0.41 and 2.34±0.35 hours was observed in 5-Fu-loaded hollow microspheres (21).

In addition it has been observed that after administration of oral drug solution, 2 out of 12 rabbits were found dead, few hours after the experiment. However, death has not been observed after microencapsulation of 5-FU in microbeads and microspheres showing decreased toxic effects. Improved efficacy and enhanced therapeutic effect of 5-FU-loaded microspheres has also been reported by Zankhana et al. (22). Similarly, efficacy of guar gum was also evaluated for colon targeting containing 5-FU by fabricating it into microspheres (23).

In the present study, cyclodextrin and 5-FU complex loaded microcarriers are prepared by utilizing dual approach that successfully involved two steps approach in a single delivery device. Firstly, the 5-FU is complexed with HP-β-CD and secondly, is encapsulation of complexed drug into polymeric carrier. Developed formulations shows promising properties of increased loading capacity, increased entrapment efficacy and provides sustained and controlled release of drug. The addition of HP-β-CD, a microbial activated biodegradable polymer add value to the formulated carriers with better ADME properties, rare toxicity and are patient friendly.

Typical characteristics of inclusion containing formulations includes faster dissolution rate and more effective absorption which translates into improved oral bioavailability of drugs with reduction in drug dosages and increased biological activity (24, 25).

Paclitaxel when complexed with cyclodextrin and loaded into nanoparticles of poly (anhydride) resulted in remarkable improvement in bioavailability of drug upto 80% (26). Bilensoy et al. (27) developed a vaginal gel loaded with 5-FU complexed with cyclodextrin to ensure long residence at the infection site with favorable drug release profile and reduced side effects. Lie et al. (28) investigated the pharmacokinetics behavior of β-cyclodextrin-oridonin complex in rats. Significant increase in drug bioavailability with enhanced solubility has been observed.

Mognetti et al. (29) prepared nanosponges containing paclitaxel-β-cyclodextrin complex that showed complete release of drug within 2 hours without causing recrystallization of drug with enhanced pharmacological activity. Dhule et al. (30) successfully evaluated liposomal curcumin’s ability against cancer. Complex of 2HP-β-CD and curcumin-liposome showed effective anticancer results both in vitro and in vivo.

At present, various approaches are being used for oral administration of 5-FU including pH sensitive polymers, enzyme dependent delivery system, prodrug approach and drug release by colonic microflora (30). Gastro-intestinal microflora is a key factor to drug delivery devices that are formulated to treat GI disorders. These microflora metabolize about 400 different types of microorganisms and several chemicals (30). A small number of microorganisms are present in stomach and upper intestine of rabbit and human as rabbits provides comparable gastrointestinal physiology and conditions with human GIT in terms of pH in different parts of GIT. So rabbits are used in this study to evaluate in vivo performance of formulated microcarriers for sustained delivery of 5-FU to treat colon disorders.

Pharmacokinetic profiling and quantification of plasma concentration

High variability in 5-FU parameters results in instability in the concentration of drugs even after administration of same drug dose. Oral use of this agent however avoid excessive side effects especially in GIT cancer. Various formulations of microspheres and microbeads were evaluated in animals which indicates suitable pharmacokinetic outcomes.
Statistical analysis

Analysis of variance showed p value less than 0.05 considered to be a significant difference in results that are mentioned in Table 2.

CONCLUSION

Various limiting factors have been observed in a conventional colon specific drug delivery systems resulting in adverse effects, incomplete release of drug at the target site and local irritation such as diarrhea. For this variety of delivery strategies and devices have been proposed for colonic drug targeting. The use of cyclodextrins and chemically modified derivatives, HP-β-CD as colon targeting carrier is an interesting approach in this regard since these oligosaccharides are neither absorbed nor hydrolyzed in the small intestine and stomach but are absorbed in large intestine. Increase in the bioavailability, expressed as change in Tmax, AUC and Cmax demonstrates that CD improves effectiveness of a given dose of therapeutic drug.

REFERENCES

Alkylating cytostatics belonging to the group of oxazaphosphorines (cyclophosphamide – CP, ifosfamide – IF) are broadly used in chemotherapy of cancer (both solid tumors and proliferative conditions of the hematopoietic system), and in pharmacotherapy of chronic inflammatory diseases and autoimmune conditions (primary systemic vasculitis, visceral lupus, periarteritis nodosa) (1, 2). General adverse effects of those agents include nausea and vomiting, alopecia, hypersensitivity reactions and myelosuppression (3). Long-term administration of CP/IF is associated with a toxic effect on gonads and sterility, and with a paradoxically increased risk of many types of cancer (prostatic, pulmonary, cystic, breast, endometrial, thyroid, hepatic, gastric) (4). Moreover, chronic use of large CP/IF doses (on the level of 60 mg/kg a day) resulted in development of myocarditis, increased arrhythmogenesis and congestive heart failure, and with interstitial pulmonary fibrosis (4, 5). In case of ifosfamide, a neurotoxic effect was also reported, manifested with signs of encephalopathy, usually disappearing following completion of the treatment (6, 7).

One of the most significant adverse drug reactions (ADR) associated with oxazaphosphorines,
and particularly with cyclophosphamide, is hemorrhagic cystitis initiated by urototoxic acrolein released in course of CP/IF metabolism. The endogenous, hepatic bio-transformation of cyclophosphamide is based mostly on cytochromes CYP2B6, CYP2C9 and CYP3A4 (8). Metabolism of cyclophosphamide with those cytochromes leads to final production of a 4-hydroxy derivative, that undergoes conversion to aldoxophosphamide releasing nitrogen mustard ñ a compound attributed the main cytostatic and alkylating properties of cyclophosphamide (8). Acrolein is also formed in course of those transformations. It is a highly reactive, α,β-unsaturated aldehyde that causes reduction of the intracellular level of glutathione and of other compounds containing thiol groups, and combining with nucleophilic compounds. Acrolein is a strong inducer of lipid peroxidation, and changing the intracellular redox balance leads to dysregulation of cytokines that participate in inflammatory processes, proliferation and apoptosis (9). In the urinary bladder the compound induced a cascade of inflammatory changes, release of numerous proinflammatory mediators and free radicals that damage the urothelium. This disturbance is characteristic particularly to cyclophosphamide, as its chemical structure makes it specifically prone to acrolein generation. A detailed pathophysiological description of oxazaphosphorine-triggered cystitis may be found in review articles (3, 10-13), including the one published by us (14). Similarly, another significant ADR characteristic for CP/IF is nephrotoxicity. Its pathogenesis is also associated with effects dependent on acrolein, synthesized with cytochromes CYP2B6 (15, 16) and CYP2C9 (17), present also in extrahepatic tissues, e.g., in kidneys. Chloroacetic aldehyde, released along with acrolein as a result of biotransformation of oxazaphosphorines in course of renal injury, is nephrotoxic, especially for proximal and collecting renal tubules. The compound intensifies the unfavorable effect of acrolein causing impairment of function of cells in renal tubules by further reduction of glutathione, acetyl-coenzyme A and ATP levels, and block of the enzymatic complex of NADH ubiquinone oxidoreductase ñ a component of the mitochondrial respiratory chain (3). Considering the fact that during its bio-transformation ifosfamide generates 40-times more acetic aldehyde than the similar dose of cyclophosphamide, it is believed that nephrotoxicity is a principal, although not sole, adverse effect of that oxazaphosphorine derivative (18).

A clinical manifestation of nephrotoxic effect of oxazaphosphorines covers a broad spectrum of disorders dependent on the type of drug (CP/IF), dose, administration route, time of therapy and some additional factors (patient age, coexistence of other nephrotoxic factors). Literature data indicate development of both glomerulopathy and tubulopathy, and even of renal failure in CP/IF-treated patients (19, 20).

Considering a variable character of renal dysfunctions and the fact that – as mentioned above – nephrotoxicity is a common ADR observed for both agents (although more characteristic for IF, as cystitis is the dominating adverse effect of CP), the aim of the study was to estimate the renal function and the level of urinary bladder injury in rats receiving a single, large dose of CP/IF.

**EXPERIMENTAL**

The medical experiment described in this paper was approved by the 1st local Ethical Commission in Krakow (No. 1/2016). The experiment was consistent with the EU Directive 2010/63 on the protection of animals used for scientific purposes and with the Polish legislation – The Law of 15 January 2015 on the protection of animals used for scientific or educational purposes (Journal of Laws, 26 February 2015, item 266).

**Test groups of animals**

Ten-week old (mature) albino Wistar rats of both sexes in equal proportions, with initial body weight of 243.3 g were used in the experiment. Animals were obtained from the Central Animal House of the Pharmaceutical Faculty of the UJCM. After being transported to the local Animal House of the Chair of Pathophysiology UJCM, animals were acclimatized for 7 days. During that time they were kept in collective cages of five animals of the same sex. Duration of acclimatization was consistent with the EU Directive 2010/63 on the protection of animals used for scientific or educational purposes (Journal of Laws, 26 February 2015, item 266).

**Plan of the experiment**

The principal part of the experiment was completed using metabolic cages allowing measurement...
of circadian diuresis and consumption of feed, in an air-conditioned pen at constant temperature of 22°C and relative humidity of 60%, with a 12/12 h day/night cycle (light phase 8 a.m. – 8 p.m; dark phase 8 p.m. – 8 a.m.). Following intraperitoneal administration of CP/IF/normal saline, respectively, to animals in groups 1, 2 and 3, study animals were placed in individual metabolic cages for 24 hours, with unlimited but monitored access to water and standard feed (Labofeed, Kcynia, Poland). Before administration of a drug/normal saline animals were weighed using a standard laboratory scales in order to determine the required CP/IF dose and placed in a tamer for rectal measurement of body temperature using a digital thermometer for rodents (Vivari, UK). Selection of the dose of 150 mg/kg b.w. for the applied nephrotoxic CP dose was consistent with literature data, recommending a single administration of 100 mg/kg b.w. (22), 150 mg/kg b.w. (23, 24), or 200 mg/kg b.w. (25, 26) as the amount causing a sub-lethal toxic effect in rats. On the same basis, the recommended single nephrotoxic dose of IF used for experimental purposes ranges between 60-240 in rabbits (27) to as much as 400 mg/kg b.w. in mice (28). Adoption of that dosage regimen results with acute urinary bladder injury developing within 4 – 24 h after CP/IF administration. Both CP and IF were purchased from Sigma Aldrich in crystalline form. Individual doses were prepared by reconstitution in 0.5 mL of normal saline and mixing with a vortex (directly before administration) of an analytically weighed dose according to the body weight of a given animal. Animals in the control group received an intraperitoneal injection with pure normal saline at the same volume of 0.5 mL, as the volume for animals in groups 1 and 2. Keeping animals in metabolic cages allowed the assessment of the following parameters within 24 h after administration of CP/IF/normal saline: body weight [g] (determined by subsequent weighing on an analytical scales), body temperature [°C] (as indicated by subsequent rectal measurement), circadian water consumption [mL/24 h], circadian feed consumption [g/24 h] and circadian diuresis [mL/24 h]. After daily urine output was measured, urine was analyzed using strip tests. Urine was subsequently centrifuged (Heraeus Instruments Megafuge 1.0 R; 2000 rpm – 719 g for 5 min), and divided into portions. Resulting samples were stored at -20°C until laboratory determinations completed with the ADVIA 1200 SIEMENS analyzer. Sodium [mM/L], potassium [mM/L], creatinine (Cr) [mM/L], uric acid [µM/L] and protein [g/L] levels were determined. Having results of circadian diuresis, circadian elimination of sodium, potassium, urea [mM/24 h], uric acid, creatinine [µM/24 h] and protein [mg/24 h] with urine was calculated. Creatinine clearance was also estimated, according to the formula:

$$CL_{cr} = \frac{Cr \text{ urine} [\mu \text{M/L}] \times \text{diuresis} [\text{mL/min}]}{\text{Cr plasma} [\mu \text{M/L}]}$$

Urea clearance was calculated according to the formula:

$$CL_{urea} = \frac{\text{urea urine} [\mu \text{M/L}] \times \text{diuresis} [\text{mL/min}]}{\text{urea plasma} [\mu \text{M/L}]}$$

Fractional excretion of sodium (FENa) was calculated using the formula:

$$\text{FENa} = \left( \frac{\text{Na urine} [\mu \text{M/L}] \times \text{Cr plasma} [\mu \text{M/L}]}{100} \right) \times \left( \frac{\text{Na plasma} [\mu \text{M/L}] \times \text{Cr urine} [\mu \text{M/L}]}{\text{Na urine} [\mu \text{M/L}] \times \text{Cr plasma} [\mu \text{M/L}]} \right)$$

The renal failure index (RFI) was calculated with the formula:

$$\text{RFI} = \left( \frac{\text{Na urine} [\mu \text{M/L}] \times \text{Cr plasma} [\mu \text{M/L}]}{\text{Na plasma} [\mu \text{M/L}] \times \text{Cr urine} [\mu \text{M/L}]} \right)$$

After the period of monitoring in metabolic cages animals were deeply anesthetized by intraperitoneal administration of 60 mg/kg b.w. of sodium pentobarbital (Morbital; Biowet, Pułały). Under general anesthesia blood was collected from the heart in amount allowing completion of all planned laboratory plasma determinations, and study rats were sacrificed by repeated intraperitoneal administration of lethal dose of sodium pentobarbital (200 mg/kg b.w.). Similarly to urine, collected blood was centrifuged and resulting plasma samples were kept frozen for the time of laboratory determinations. Sodium, potassium, urea [mM/L] and creatinine (Cr) and uric acid [µM/L] levels were determined in plasma with the same analyzer as the one used for urine samples. Protein was not assayed in plasma. After definitive cease of vital signs, cystectomy and nephrectomy was performed in study animals in order to obtain tissue necessary for subsequent histopathological assessment of kidneys and urinary bladders.

**Urine analysis with strip tests**

Strip tests (Insight, ACON Laboratories), allowing pH and specific gravity (SG) assessment, glucose level, presence of blood, leukocytes, nitrites, urobilinogen, ketones, bilirubin and protein, were used for qualitative and semi-quantitative assessment of urine obtained from study animals. To perform a test, strips were placed in previously mixed urine samples, excess was filtered out and strips left for 60 s (according to the manufacturer’s instructions after that time the result of blood and protein presence could be considered reliable). After that time strips were compared to the scale printed on the package and results were recorded.
Histological assessment of collected urinary bladders and kidneys

The histological analysis was performed at the Department of Anatomo-pathology of the Non-public Healthcare Institution „Prosmed” in Kraków. Kidneys and bladders collected in course of the post-mortem examination were washed in normal saline and fixed for 24 hours in 8% formal in phosphate buffer solution (PBS pH 7.4). Collected specimens were subsequently washed under running water for 2 h, and dehydrated in ethanol (condensations increasing from 50 to 100%). Before embedding in paraffin, specimens were passed through xylene solution in order to be cleared. From xylene, specimens were transferred to a 1 : 1 xylene and paraffin mixture and incubated at 37°C for 2 h. Then, individual tissue fragments were transferred twice into pure paraffin and incubated at 62°C. After 2 h, specimens were embedded into paraffin blocks. After setting down blocks were sliced with a microtome, and resulting sections were dried on a glass slide at 37°C. Specimens were stained with hematoxilin-eosin (HE) in order to facilitate the histological assessment of inflammation. Specimens of urinary bladders and kidneys from animals receiving normal saline (control group 3) were compared to those from animals treated with CP/IF.

The histological analysis was performed using an optical (light) microscope (Delta Optical) at a magnification of 40 × (urinary bladders) and 100 × (kidneys). The microscopic images were taken using a DLT-Cam Basic 2MP microscopic camera and DLTCamViewer software.

Statistical analysis

Results of qualitative and semi-quantitative urine analysis completed with strip tests were not subject to statistical assessment. Obtained results of vital signs and laboratory determinations of urine and plasma were initially assessed using the Shapiro-Wilk test, in order to verify normality of distribution. With verified normality of distribution, the essential statistical analysis of differences between groups (group 1 vs. group 3, and group 2 vs. group 3) was performed using the t-Student test, with the accepted statistical significance level of p ≤ 0.05. In those cases when results verified by the initial Shapiro-Wilk test failed to meet criteria of a normal distribution, differences were tested with the Mann-Whitney test, also with the statistical significance level of p ≤ 0.05.

Table 1. Metabolic cage measurement - results (mean ± SD) of the living parameters of the animals used in the experiment.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 CP-treated rats</th>
<th>Group 2 IF-treated rats</th>
<th>Group 3 Control rats</th>
<th>Statistical analysis (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight[g]</td>
<td>248.50 ± 32.30</td>
<td>257.00 ± 10.89</td>
<td>226.6 ± 44.89</td>
<td>NS</td>
</tr>
<tr>
<td>Body temperature[°C]</td>
<td>36.23 ± 0.90</td>
<td>35.50 ± 0.25</td>
<td>37.43 ± 0.93</td>
<td>0.04 0.01</td>
</tr>
<tr>
<td>Daily water intake[mL/24h]</td>
<td>22.50 ± 11.47</td>
<td>13.50 ± 7.19</td>
<td>26.34 ± 4.07</td>
<td>NS 0.02</td>
</tr>
<tr>
<td>Daily feed intake[g/24h]</td>
<td>1.98 ± 1.37</td>
<td>4.53 ± 3.72</td>
<td>23.75 ± 6.16</td>
<td>&lt; 0.001 &lt; 0.001</td>
</tr>
</tbody>
</table>

NS - Non-significant

Table 2. The results of the estimated plasma parameters (mean ± SD) in study rats.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 CP-treated rats</th>
<th>Group 2 IF-treated rats</th>
<th>Group 3 Control rats</th>
<th>Statistical analysis (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na [mM/L]</td>
<td>141.95 ± 1.26</td>
<td>144.75 ± 1.95</td>
<td>142.06 ± 1.79</td>
<td>NS 0.04</td>
</tr>
<tr>
<td>Urea [mM/L]</td>
<td>8.97 ± 1.54</td>
<td>10.93 ± 2.12</td>
<td>6.34 ± 0.75</td>
<td>0.03 &lt; 0.001</td>
</tr>
<tr>
<td>Creatinine [µM/L]</td>
<td>38.45 ± 7.96</td>
<td>34.67 ± 7.48</td>
<td>28.85 ± 1.77</td>
<td>0.05 0.05</td>
</tr>
<tr>
<td>Uric acid [µM/L]</td>
<td>165.82 ± 45.77</td>
<td>178.38 ± 21.88</td>
<td>157.96 ± 35.14</td>
<td>NS NS</td>
</tr>
</tbody>
</table>

NS - Non-significant
RESULTS

Vital signs

After the end of monitoring in metabolic cages, body weight of study animals was not significantly different. 24 h after administration of a drug, animals receiving CP or IF had a significantly lower body temperature compared to control animals. Similarly, a statistically significant reduction of circadian feed consumption was found in both groups 1 and 2 compared to the control, which was probably a result of unfavorable systemic effect of CP/IF, particularly on gastrointestinal mucosa. Moreover, rats receiving IF consumed a significantly lower amount of water within 24 hours post the drug administration, compared to the control.

Detailed results of measurement of vital signs are presented in Table 1. Considering the fact that measurements were characterized by a normal distribution, the statistical analysis of intra-group differences was performed using the t-Student test.

Qualitative and semi-quantitative urinalysis with strip tests

Values of pH and specific gravity are described below, along with description of parameters measured in urine. A minor leukocyte count (estimated at 15 (±); n = 7) and a minor proteinuria estimated at (+) (30 [mg/dL]; n = 5) were demonstrated in control animals. Absence of other measured components was confirmed in their urine.

Urine from animals receiving CP was characterized by leukocytosis in 25% of cases estimated at + (70 [leu/µL]), in 25% at ++ (125 [leu/µL]), and in the remaining half of cases at +++ (500 [leu/µL]). Presence of blood was confirmed in all urine samples, estimated at +++. Similarly, proteinuria was found in all animals in the group 1, estimated at +++ (300 [mg/dL]). Nitrites, urobilinogen, ketones, bilirubin or glucose were found in urine from none of CP-treated animals.

Strip urinalysis of rats treated with IF gave similar results: no urobilinogen, ketones, bilirubin or glucose were found, but presence of nitrites was demonstrated in 25% of cases. Leukocytosis was lower than that measured in the group 1: in 25% of cases it was estimated at +/- (15 [leu/µL]), in 25% at + (70 [leu/µL]), and no leukocytes were found in urine in the remaining half of cases. Presence of blood estimated at +++ was demonstrated in half of cases; but no hematuria was found in the other half. Similarly to CP-treated animals, rats treated with IF demonstrated proteinuria, estimated at +++ (300 [mg/dL]) in all cases.

Table 3. The results of the estimated urine parameters (mean ± SD) in study rats.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 CP-treated rats</th>
<th>Group 2 IF-treated rats</th>
<th>Group 3 Control rats</th>
<th>Statistical analysis (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diuresis [mL/24 h]</td>
<td>16.70 ± 8.31</td>
<td>8.95 ± 7.49</td>
<td>5.85 ± 2.37</td>
</tr>
<tr>
<td></td>
<td>Diuresis [mL/min]</td>
<td>0.012 ± 0.006</td>
<td>0.006 ± 0.005</td>
<td>0.004 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.75 ± 0.29</td>
<td>6.13 ± 0.75</td>
<td>8.94 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>SG [g/cm³]</td>
<td>1.014 ± 0.003</td>
<td>1.023 ± 0.006</td>
<td>1.011 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Na [mM/L]</td>
<td>46.00 ± 10.94</td>
<td>80.83 ± 36.08</td>
<td>127.58 ± 34.67</td>
</tr>
<tr>
<td></td>
<td>K [mM/L]</td>
<td>68.10 ± 27.31</td>
<td>159.32 ± 81.20</td>
<td>352.96 ± 64.66</td>
</tr>
<tr>
<td></td>
<td>Urea [mM/L]</td>
<td>441.35 ± 115.32</td>
<td>949.25 ± 532.44</td>
<td>1003.96 ± 188.91</td>
</tr>
<tr>
<td></td>
<td>Creatinine [mM/L]</td>
<td>3.35 ± 1.17</td>
<td>6.75 ± 4.34</td>
<td>6.10 ± 1.10</td>
</tr>
<tr>
<td></td>
<td>Uric acid [µM/L]</td>
<td>826.18 ± 321.76</td>
<td>1378.10 ± 401.61</td>
<td>1286.54 ± 296.60</td>
</tr>
<tr>
<td></td>
<td>Urine protein [g/L]</td>
<td>2.5 ± 0.92</td>
<td>3.91 ± 1.15</td>
<td>0.81 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>Na [mM/24 h]</td>
<td>0.78 ± 0.44</td>
<td>0.55 ± 0.14</td>
<td>0.75 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>K [mM/24 h]</td>
<td>1.09 ± 0.49</td>
<td>1.03 ± 0.20</td>
<td>2.05 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>Urea [mM/24 h]</td>
<td>6.36 ± 1.91</td>
<td>5.72 ± 0.61</td>
<td>5.80 ± 2.38</td>
</tr>
<tr>
<td></td>
<td>Creatinine [µM/24 h]</td>
<td>50.49 ± 18.12</td>
<td>42.21 ± 13.76</td>
<td>35.19 ± 13.85</td>
</tr>
<tr>
<td></td>
<td>Uric acid [µM/24 h]</td>
<td>13.53 ± 6.82</td>
<td>10.58 ± 5.36</td>
<td>7.49 ± 3.75</td>
</tr>
<tr>
<td></td>
<td>Urine protein [mg/h]</td>
<td>38.70 ± 17.12</td>
<td>23.57 ± 14.83</td>
<td>5.27 ± 4.47</td>
</tr>
</tbody>
</table>

NS - Non-significant, SG - specific gravity
Parameters determined in plasma

Considering signs of hemolysis evident in majority of plasma samples, results of plasma potassium levels had been discarded as non-reliable.

Values of plasma parameters assessed in CP-treated were not significantly different from control animals, except for a statistically significant increased urea and creatinine levels. But IF-treated animals were characterized with a significant hypernatremia, increased plasma urea and creatinine levels, with a statistically insignificant reduction of the plasma uric acid level. Detailed results of measurement of plasma parameters are presented in Table 2. Considering the fact that measurements had no normal distribution, the statistical analysis of intra-group differences was performed using the Mann-Whitney test.

Parameters determined in urine

The circadian diuresis and the calculated minute diuresis was significantly higher in CP-treated animals. On the contrary, IF-treated rats demonstrated no significant difference in diuresis compared to control animals. In both groups of treated animals a significant acidosis of urine was found, compared to the control group. Specific gravity of urine from rats receiving IF was significantly higher compared to the control.

Urine levels of all determined substances were significantly lower in the group 1 (CP treatment) compared to the control, except for protein. Protein levels were statistically significantly higher in CP-treated animals. IF-treated rats were characterized by significantly lower and higher of potassium and protein levels, respectively, compared to the control.

The analysis of circadian urine elimination of tested substances demonstrated in both groups of treated animals a statistically significant reduction of potassium elimination and a significant proteinuria. Other urine components were eliminated in amounts similar to those observed in the control group.

Detailed results of measurement of plasma parameters are presented in Table 3. Considering the fact that measurements were characterized by a normal distribution, the statistical analysis of intra-group differences was performed using the t-Student test.

Values of calculated parameters: creatinine clearance, urea clearance, fractionated sodium elimination and renal failure index were not significantly different in CP-treated rats compared to the control. On the contrary, comparing IF-treated animals with the control, a statistically significant reduction of urea clearance, FENa and RFI were found. Creatinine clearance was an exception.

The above mentioned relationships are illustrated in Figures 1-4, below (graphs present median values, quartiles and ranges for individual variables).

Figure 1. Creatinine clearance in cyclophosphamide-treated (left) and ifosfamide-treated (right) rats (no statistically significant differences revealed in both comparisons)
Nephrotoxicity of a single dose of cyclophosphamide and... 1585

Results of histopathological analysis of urinary bladders and kidneys

Histopathological assessment of urinary bladder specimens from rats receiving a single large dose of CP demonstrated congestion with inflammatory infiltration in stroma of the cystic mucosa, and epithelium with signs of regeneration. Moreover, clusters of dilated tubules covered by a regular cubic epithelium and a low cylindrical epithelium were visible in selected specimens. Urinary bladders of animals treated with a single large dose of IF presented similar disturbances to those observed in CP-treated rats, with additional signs of exfoliation of the lining epithelium.
Histopathological analysis of kidneys demonstrated minor congestion in both CP and IF-treated animals, but besides that the presentation was within the normal range determined for control animals. The samples of microscopic images of the urinary bladders and kidneys obtained from control and CP/IF treated rats are shown in Figures 5 and 6, respectively.

DISCUSSION

The experiment demonstrated existence of the following principal disorders:

1. Rats treated with a single dose of CP demonstrated reduced body temperature and reduced circadian feed consumption, with increased diuresis and decrease of urine pH. That group presented a reduced circadian elimination of potassium and a significant proteinuria, as well as increased plasma creatinine and urea levels, compared to control animals.

2. Similarly to CP-treated rats, animals receiving a single dose of IF were also characterized by reduced body temperature and reduced circadian feed consumption, and by urine pH decrease. Contrary to CP-treated animals, that group presented diuresis comparable to that observed in control animals, with a lower daily water consumption. Administration of a single dose of IF resulted in reduced 24-hour elimination of potassium and a significant proteinuria, just like in case of a single dose of CP. Similarly to animals treated with CP, administration of IF resulted in increased creatinine and urea plasma levels. Moreover, IF-treated animals presented reduced urea clearance values, fractionated sodium elimination and acute renal failure index.

Although being statistically significant, reduced urine levels of all parameters determined in CP-treated animals compared to the control group, were rather a result of increased diuresis in that group of rats, and not a result of a real increase of urine elimination of those substances. Particularly, the analysis of circadian elimination of electrolytes and nitrogen compounds demonstrated a reduced urine elimination of potassium only, with a significant proteinuria in CP-treated animals (and in IF-treated, as well). Considering those laboratory results globally, it should be stated that renal dysfunctions observed after administration of both CP and IF were of functional character and were not accompanied by any morphological injury of kidneys evident in the histopathological assessment. Therefore, a single exposure to oxazaphosphorines was sufficient to cause a functional disorder of kidneys, manifested principally by impaired tubular transport in terms of secretion and reuptake of potassium, and – in case of CP-treated animals – by impaired condensation of urine. Decreased urine potassium elimination may be

![Figure 4. Renal failure index (RFI) in cyclophosphamide-treated (left) and ifosfamide-treated (right) rats (p < 0.05 for Control-IF rats, non-significant for Control-CP ones)](image-url)
associated with an expected retention of the element in blood. That assumption could not be verified because of hemolysis of plasma samples. Regulation of plasma potassium levels (and consequently the dynamics of its elimination with urine) is based mostly on exchange of the ion between intra- and extracellular fluid. Kidneys are a critical organ engaged in regulation of systemic amount of potassium. Within a nephron, potassium ions undergo glomerular filtration, and are subsequently reuptaken in proximal tubules and in the loop of Henly. The final urinary elimination of majority of potassium ions depends on the active secretion of the ion by cells of distal and collecting tubules, determined by increased plasma levels of the electrolyte, increased Na⁺. K⁺/H⁺ exchange with participation of the aldosterone-regulated ATPase in basal-lateral membrane and a direct secretion of the ion into urine (29, 30). Considering a reduced circadian potassium elimination observed in study animals, a thesis may be formulated that both CP and IF contribute to dysfunction of distal and collecting tubules.

Urine acidification is an additional argument supporting tubulopathy caused by CP/IF. The process is also associated with potassium metabolism, as mentioned above (K⁺/H⁺ exchange). In general terms it may be stated that two processes occur in nephrons: reuptake of alkalis and secretion of acids. The first one takes place in proximal tubules, and the other one in distal tubules (31). Secretion of H⁺ ions into urine takes place via the mentioned above antiport K⁺/H⁺ - ATPase and the luminal pump H⁺ – ATPase, and a final acidification of urine depends also on phosphate and ammonia buffer (32, 33). It seems that CP and IF may impair those mechanisms, leading to final reduction of urine pH and increased potassium reuptake.

Moreover, CP-treated animals demonstrated polyuria, which – in face of circadian water consumption comparable to that observed in the control group – may constitute another argument in favor of dysfunction of distal and collecting tubules. Reuptake of water and final condensation of urine depends on aquaporins – integral proteins of water
channels induced in apical membranes of collecting tubules by antidiuretic hormone (34). Therefore, polyuria observed in the group of rats receiving a nephrotoxic dose of CP may be a result of impairment of that mechanism. That observation is contrary to literature reports indicating a possible development of „syndrome of nephrogenic syndrome of inappropriate antidiuresis” induced by CP as a result of increased expression and hyperactivity of aquaporins in collecting tubules of rats following administration of the dose of 12-96 mg/kg b.w. (35) or 25-50 mg/kg b.w. of CP (36), in 12-72 h after administration of cyclophosphamide. The quoted studies indicate also that the effect may be ADH-independent, and time- and dose-dependent, therefore the effect of CP on the volume of diuresis has to be more precisely defined.

A significant proteinuria was demonstrated in both treated groups. Pathogenesis of that disorder seems complex and may be a result of above mentioned dysfunction of renal tubules, or be a consequence of other disorders. According to the general pathophysiological classification, proteinuria may be of prerenal character (overload), renal character (glomerular or tubular) or extrarenal character (e.g., inflammatory, neoplastic or obstructive disturbances of the lower urinary tract) (37, 38). Therefore, presence of increased urinary elimination of protein in study rats may be a result of impairment of resorptive function of tubules, but considering presence of oxazaphosphorines administration were reported — a combination of glomerular, proximal or distal tubular damage, Fanconi syndrome and reversible AKI or a permanent chronic kidney disease were revealed. These disturbances are characterized by a considerable variability in the onset, clinical manifestation, severity and reversibility and dependent on the dose and time of the therapy (18-20).

Renal dysfunctions observed in study animals are consistent with the above mentioned literature data. Described disorders developing as a result of administration of a single, nephrotoxic dose of CP/IF also suggest a dysfunction of mostly distal and collecting tubules, with absence of morphological changes in kidneys. Proteinuria, urine acidification with reduced circadian elimination of potassium and increased plasma levels of creatinine and urea were demonstrated as a result of a single exposure to nephrotoxic doses of CP/IF. The commonly recognized lower nephrotoxicity of cyclophosphamide was also confirmed in this study. No laboratory features of AKI (RFI, FE\text{Na}) were demonstrated following administration of a single dose, contrary to IF-treated rats. However, a significant polyuria was observed in case of CP, in place of the literature-reported antidiuresis syndrome, which may be a result of different methodology of the study, different dosage and observation time.

CONCLUSIONS

Multiple renal dysfunctions resulted from oxazaphosphorines administration were reported — a combination of glomerular, proximal or distal tubular damage, Fanconi syndrome and reversible AKI or a permanent chronic kidney disease were revealed. These disturbances are characterized by a considerable variability in the onset, clinical manifestation, severity and reversibility and dependent on the dose and time of the therapy (18-20).
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The dangers of addiction are generally well-known, nevertheless people continue to start taking drugs. Dependence on drugs and alcohol is assigned to the most common brain disorders. Almost 50% of all persons who have applied for reliance on drug therapy as the primary drug used opioids. Therefore, researchers are interested in naturally occurring psychoactive alkaloid ibogaine, found in African plant *Tabernanthe iboga* Baill. (Apocynaceae). Psychoactive properties of ibogaine have been known for decades (1).

There are published data that ibogaine can facilitates the symptoms of abstinence (2). In the late 20th century, many scientific researches started to be conducted with an aim to find out the mechanism of action of ibogaine. The pharmacology of ibogaine is quite complex, affecting many different neurotransmitter systems simultaneously. Non-clinical studies have demonstrated that ibogaine reduces craving for cocaine and morphine, attenuates morphine withdrawal symptoms (3). However, the pharmacological targets underlying the physiological and psychological actions of ibogaine are not completely understood (4). The identified antagonistic activity of ibogaine on N-methyl-D-aspartate receptors as well as its agonist activity on opioid receptors can be regarded as a possible mechanism of anti-addictive action (5).

Ibogaine is metabolized by cytochrome P4502D6 (CYP2D6) into active metabolite noribogaine (12-hydroxyibogamine) (6), which can condition the long-term effect of ibogaine (7, 8).
Although they share similar chemical structures, ibogaine and noribogaine display different binding profiles (9). Although both alkaloids increase extracellular 5-HT in the brain, noribogaine is more potent in this respect (10). Previous study reports, that noribogaine is less toxic (11) and can be safer and causing less undesirable reactions as compared with parent drug (10). However, usually noribogaine is analyzed after administration of ibogaine while we investigated the pharmacokinetic parameters of noribogaine after administration of both – ibogaine, as well as noribogaine.

In previous study (12) we calculated the amounts of tested substances in organs (heart, liver, spleen, brain, kidney and muscle) of mice after ibogaine and noribogaine administration directly into the mice stomach via the stomach tube. In present study, we evaluated the main pharmacokinetic parameters of ibogaine and noribogaine in plasma and organs of mice.

EXPERIMENTAL

Animals

The experiments were conducted with non-linear white laboratory mice (20–25 g) of the age of 4 to 6 weeks in compliance with the Law on the Care, Keeping and Use of the Animals of the Republic of Lithuania. The animals were kept in compliance with the requirements of the Good Laboratory Practice (GLP). The experiments were performed in cooperation with the Laboratory of Molecular Neurobiology of the Institute of Neurosciences of the Medical Academy of the Lithuanian University of Health Sciences. Adult male mouse (n = 54) weighing 20-25 g were housed individually in rooms maintained at ~20°C, with a regular light-dark cycle. Water was available in the living cages at all times. Permission for experiments with Laboratory Animals No. 0172 was issued by the State Veterinary Authority of Lithuania.

Chemicals and reagents

Ibogaine hydrochloride, gradient purity eluents and fluorescein sodium salt (internal standard) purchased from manufacturer “Sigma-Aldrich Chemie” (Switzerland) were used for the researches. Noribogaine base was kindly supplied by Reform Italia (Endine, Italia). Ibogaine and noribogaine were stored protected from light. Water purified by Millipore water purification system (Millipore, Bedford, USA) was used for the experiments. Oasis HLB cartridges (30 mg sorbent, size of particles – 30 µm) were supplied by Waters (Milford, USA). Blank human plasma was received from the Division of Haematology of the Clinics of the Lithuanian University of Health Sciences. Heparin sodium salt was purchased from UAB “Labochema LT”.

Pharmacokinetic experiments

Mice were randomly assigned to three groups – ibogaine group, noribogaine group and control group. Ibogaine and noribogaine solutions have been prepared ex tempore prior to each test by dissolving accurately weighed quantities of the sub-
stance in deionized water. The volume of solution was recalculated in accordance with the weight of each mouse (e.g., for the mouse of the weight of 25 g – 0.25 mL of prepared solution). Single dose of ibogaine (26.3 mg/kg) and noribogaine (31.5 mg/kg) were administered intragastrically to mice via a specially designed stomach tube. Control mice received the same amount of saline. The same method of administration was used.

Animals were dislocated and decapitated 15 min, 30 min, 2 h, 4 h, 6 h, 8 h, 16 h, 24 h, 48 h after the intragastric administration of substance. Three mice were decapitated in each time interval. The blood was collected to centrifugal tube with 25 µL of heparin, centrifuged and collected blood plasma was frozen in the temperature of -40°C. Prepared internal organs (liver, kidney, brain, heart, spleen and muscle) were frozen in the temperature of -40°C until the time of assay by high performance liquid chromatography (HPLC).

Ibogaine and noribogaine concentrations were determined by HPLC with fluorescence detector. Solid-phase extraction (SPE) was used for the removal of proteins and interfering components. Analytical procedure from plasma and organs and chromatographic analysis were described in previous study (12). The HPLC method validation parameters in our study were specificity, precision

Figure 2. Chromatograms obtained from liver (A), spleen (B), heart (C), kidney (D), brain (E), muscle (F) of tested and control mice. Peak 1 – noribogaine, peak 2 – ibogaine, peak 3 -fluoresceine
(intermediate precision), linearity and lower limit of quantitation. The specificity of the method was investigated by analyzing three different batches of blank human plasma samples. It shows, that peaks in blank plasma did not impede the identification of the tested materials (Fig. 1).

The same results were obtained analyzing internal organs of tested and control mice (Fig. 2). RSD were calculated: it was 5.1% for noribogaine and 6.9% for ibogaine. The LLOQs (lower limit of quantitation) were 1.4 ng/mL for ibogaine and 2.15 ng/mL for noribogaine. The correlation coefficients (r) for calibration curves were equal to or better than 0.98 (12).

Pharmacokinetic parameters were calculated by using pharmacokinetic program “Kineticca” (4.0 version, USA). Calculated pharmacokinetic parameters include the following: volume of distribution (Vd), area under curve (AUC), elimination half-life (T1/2), maximum concentration (Cmax). Pharmacokinetic (AUCint, Cmax, Tmax, T1/2) variables were analyzed by non-compartmental model following a trapezoidal rule.

RESULTS
Pharmacokinetic profiles in plasma
Table 1 summarizes the pharmacokinetic parameters for ibogaine and noribogaine after intragastrically administration of ibogaine (26.3 mg/kg) or noribogaine (31.5 mg/kg). Noribogaine was detected in plasma and organs after administration of either ibogaine or noribogaine. The metabolite was detected at the earliest time point (15 min), consistent with first pass metabolism of the parent drug (13).

Figure 3 illustrates the time-concentration profiles for ibogaine and noribogaine in blood plasma. Following intragastric administration circulating levels of ibogaine and noribogaine peaked at 30 min (Tmax) after ibogaine administration. Noribogaine Cmax (185 ± 0.02 ng/mL) was much less than that of ibogaine (475 ± 0.05 ng/mL), inconsistent with previous reports (14), giving a noribogaine-to-ibogaine Cmax ratio of 0.39. These data show, a much smaller fraction of ibogaine is metabolically converted to noribogaine when ibogaine is administered intragas-

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Table 1. Basic pharmacokinetic parameters of ibogaine and noribogaine in plasma of mice.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Ibogaine after ibogaine</th>
<th>Noribogaine after ibogaine</th>
<th>Noribogaine after noribogaine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmax (h)</td>
<td>0.5 ± 0.015</td>
<td>0.5 ± 0.01</td>
<td>4 ± 0.02</td>
</tr>
<tr>
<td>Peak plasma concentration Cmax (ng/mL)</td>
<td>475 ± 0.05</td>
<td>185 ± 0.02</td>
<td>150 ± 0.02</td>
</tr>
<tr>
<td>Elimination half-life T1/2 (h)</td>
<td>1.95 ± 0.11</td>
<td>4.42 ± 0.09</td>
<td>6.14 ± 0.21</td>
</tr>
<tr>
<td>Volume of distribution Vd (mL/mg)</td>
<td>0.04 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td>Clearance CL (mL/h/mg)</td>
<td>0.015 ± 0.00</td>
<td>0.03 ± 0.001</td>
<td>0.04 ± 0.001</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD values from individual animals (n = 3) assayed in duplicate

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Figure 3. Time-concentration profiles for ibogaine (IBO) and noribogaine (NOR) in plasma of mice after intragastric administration of ibogaine (26.3 mg/kg) or noribogaine (31.5 mg/kg) (n = 3)
Plasma levels of ibogaine were undetectable 24 h, but plasma levels of noribogaine were 2.5 ± 0.01 ng/mL after intragastrically administration of ibogaine. The mean Vd of ibogaine in plasma was 0.04 ± 0.01 mL/mg body weight and the mean T1/2 was 1.95 ± 0.11 h in agreement with previous reports (10, 15). The mean Vd of noribogaine after ibogaine administration was 0.20 ± 0.01 mL/mg and T1/2 was 4.42 ± 0.09 h.

Circulating levels of noribogaine increased slowly to a peak (150 ± 0.02 ng/mL) at 4 h (T max) after intragastric administration of noribogaine. Plasma levels of noribogaine decreased rapidly but it was 2.5 ± 0.01 ng/mL 24 h after noribogaine administration. The mean Vd of noribogaine was 0.37 ± 0.05 mL/mg body weight and the mean T1/2 was 6.14 ± 0.21 h.

Pharmacokinetic profiles in organs

The main data of pharmacokinetic parameters for ibogaine and noribogaine in organs of mice are presented in Table 2. The results demonstrate that ibogaine and noribogaine are rapidly detected in organs following intragastrically administration of ibogaine or noribogaine. Four hours after intragastrically administration to mice, ibogaine was found in high concentration in spleen (4.88 ± 0.11 ng/mg) and the lower in brain (0.3 ± 0.02 ng/mg). The longest total systemic exposure (AUCtot) was to spleen (134.54 ± 34.49 ng◊h/mg) and the smallest – to muscle (2.75 ± 0.40 ng◊h/mg) and brain (3.55 ± 0.31 ng◊h/mg).

The highest concentration of noribogaine was found in the spleen too (15.49 ± 0.08 ng/mg) after ibogaine administration and in the liver (15.55 ± 0.09 ng/mg) after administration of noribogaine. The lowest levels of noribogaine were in heart after ibogaine or noribogaine administration. The longest total exposure of noribogaine calculated to spleen (97.23 ± 0.16 ng◊h/mg) after ibogaine administration and to liver (97.92 ± 1.44 ng◊h/mg) after administration of noribogaine. Whereas the smallest were to muscle (9.28 ± 0.54 ng◊h/mg), brain (9.48 ± 0.29 ng◊h/mg) and heart (9.57 ± 1.34 ng◊h/mg) after ibogaine and to muscle (14.05 ± 0.41 ng◊h/mg) and spleen (15.29 ± 0.57 ng◊h/mg) after noribogaine administration.

DISCUSSION AND CONCLUSIONS

It is known that administration of ibogaine to primates leads to formation of metabolite – noribogaine (7, 8). The circulating time and concentration of metabolite noribogaine depend on route of administration of ibogaine. It is estimated that a substantial fraction of ibogaine is metabolically converted to noribogaine when ibogaine is given via the i.p. route and much smaller fraction of ibogaine is converted to noribogaine after i.v. administration of ibogaine (10). The present results demonstrate, that
noribogaine $C_{\text{max}}$ was lower than that of ibogaine after intragastrically administration of ibogaine, inconsistent with previous reports (14, 16). Previous studies have shown that the ratio of noribogaine to ibogaine in the bloodstream is much higher when ibogaine is injected by the i.p. route rather than i.v. route (10). We observed that the ratio of noribogaine to ibogaine after intragastrically ibogaine administration is higher rather after i.v. injection but lower rather i.p. injection. Comparing our results with literature data, we can conclude that the peak concentration of noribogaine is achieved faster ($T_{\text{max}}$, 0.5 h) when ibogaine is administrated intragastrically rather than the i.p. route ($T_{\text{max}}$, 2.4 h) or i.v. route ($T_{\text{max}}$, 2.2 h) (10).

Our results demonstrate that volume of distribution (Vd) of noribogaine reaches a higher values than ibogaine after intragastric administration of either ibogaine or noribogaine. Low values of half-life and Vd of ibogaine show the faster elimination from plasma in agreement with previous reports (17) indicating that long-lasting effect of ibogaine attributable to metabolite (7, 18).

However, a major aim of the present study was to compare the pharmacokinetic properties of noribogaine after administrations of ibogaine and noribogaine. Our results demonstrate that noribogaine achieves higher concentration in a shorter period of time in plasma after administration of ibogaine rather than noribogaine itself inconsistent with report of Zubaran et al. (14). However, peak concentration occurs at 2-3 h and slow elimination of noribogaine was established in previous reports too (19). Noribogaine is less distributed and elimination is faster after administration of ibogaine. Thus, noribogaine may be more potent in the treatment of drug dependence when noribogaine is administered itself rather than ibogaine although its absorption is longer.

Data presented in the literature show that pharmacokinetic parameters depend on different route of administration and dose of substance. After i.p. and s.c. injection of ibogaine in rats, the highest level of the substance is achieved in brain and adipose tissue one hour after administration (18). Whereas present results demonstrate high concentration of ibogaine in spleen 4 hours, and in liver 30 min after intragastrical administration.

Present data in brain tissue show that ibogaine and noribogaine penetrate the blood-brain barrier in agreement with previous reports (10, 13, 14). Noribogaine achieves higher concentration in brain tissue compared with ibogaine after ibogaine administration. However, the concentration of noribogaine in brain are greater after administration of noribogaine than of ibogaine. The concentrations of ibogaine and noribogaine have been measured in rat brain following both oral and i.p. administrations (40 mg/kg i.p., 50 mg/kg per os) (13, 14, 16). The results for concentrations of noribogaine in lower brain regions (cerebellum and brainstem) after administration of ibogaine were lower, compared to ibogaine. However, the concentrations of noribogaine in the higher regions of the brain (cortex and striatum) were higher after intragastric administration of either ibogaine or noribogaine. The concentrations of noribogaine in all regions of brain were much greater after administration of noribogaine than after ibogaine (13, 14).

Literature data show, that ibogaine can lead to serious cardiac-rhythm abnormalities (20-24). So, we compared total systemic exposure in heart to ibogaine and noribogaine. High values of $\text{AUC}_{\text{tot}}$ allow to agree with these statements. Furthermore, high value of $\text{AUC}_{\text{tot}}$ of noribogaine suggests, that noribogaine can lead this abnormalities too. Unfortunately, additional experiments are needed to determine effects of noribogaine on the cardiovascular system. Although it is believed that noribogaine constitutes the major cardiac risk after ibogaine intake (17).

Interestingly, concentration of ibogaine and noribogaine increases in spleen, liver and brain, while concentration in blood plasma is already declining. Ibogaine is sequestered in fat (18). Another depot might be the platelets or other blood components, as concentrations of ibogaine were higher in the whole blood than in plasma (25). It could affect the results of our study, whereas we tested plasma of mice (which does not contain the platelets), while spleen and liver are tissues of higher blood perfusion (i.e., platelets can affect the results of ibogaine and noribogaine concentrations). Partitioning of the parent drug into brain lipid may serve as a slow release storage “depot” (13). It can lead to concentration of ibogaine increasing in the brain even when the concentration in plasma already declines.

We observed that noribogaine $C_{\text{max}}$ exceed that of ibogaine in liver, kidney and brain of mice after ibogaine administration. This finding is consistent with the conversion of ibogaine to noribogaine via first-pass metabolism in the liver, as previously reported (6, 10, 26). Noribogaine $C_{\text{max}}$ (7.32 ± 0.12 ng/mg in kidney, 1.95 ± 0.14 ng/mg in brain) exceeded that of ibogaine (1.25 ± 0.09 ng/mg in kidney, 0.3 ± 0.02 ng/mg in brain) to yield a noribogaine-to-ibogaine $C_{\text{max}}$ ratio of 5.86 in kidney and 6.5 in brain. These results confirm the hypothesis
that ibogaine is \textit{o}-demethylated to noribogaine in brain \cite{25} and possibly in kidney. However, more further studies are needed to estimate this.

The present results demonstrate a widespread distribution of ibogaine and noribogaine throughout the body after ibogaine and noribogaine administration. However, some of the results are difficult to explain, for example low values of SD for $C_{\text{max}}$ or long absorption of noribogaine after administration of noribogaine. It may be affected by our chosen non-compartment model and low number of animals. Although an increasing number of pharmacokinetic studies of ibogaine and noribogaine are in scientific databases \cite{26, 27}, but different results lead us to extend studies using two-compartment or even multi-compartment model and compare the results.

High concentration and AUC$_{\text{tot}}$ of noribogaine in brain could be more efficacious alternative to ibogaine as a medication for the treatment of addiction. High values of AUC$_{\text{tot}}$ in heart samples may determine the long elimination from this organ. So, further studies, can noribogaine as ibogaine lead to cardiovascular abnormalities, are needed. Noribogaine $C_{\text{max}}$ ratio with ibogaine $C_{\text{max}}$ in kidney and brain after ibogaine administration may also result in metabolism of alkaloid in these organs.

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General

Law and Economics of Branded-to-Generic, Generic-to-Generic and Generic-to-Branded Substitution Process in Poland

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Abstract: Polish setting is an example of two extreme-sides models of a market in pharmaceutical background – market and central mechanism. Unfortunately, absence of vital institutional and social infrastructure understood as law that serves the goal of system efficiency, leads to leak of prognostic conflicts solving, lack of control over the public funds and lack of sustainable development. Substitution is a process that influences patients, pharmacists, marketing authorization holders and healthcare system in general. Pharmacy under Polish law is a place of drug dispensing not yet a place, where adequate healthcare protection (pharmaceutical) services are carried. The intention of the paper is to objectively understand the law effects in this background and raise voices – authors take the narrative view and do not advocate for any stakeholder of the system. Therefore, through an international discussion, respecting the trend of globalization and consolidation of legacy systems into global drug safety system, the goal of influencing the long-term shape of an institutional and social harmony within the process of drug substitution in Poland is to be stressed through this paper.

Keywords: community pharmacy, drug substitution, economics, legislation, reimbursement, pharmacy law

Under the Pharmaceutical Law Act of 6 September 2001 (1) (Ph. Law Act) pharmacy is a healthcare protection facility in which entitled persons provide specified pharmaceutical services (Art. 86). Community pharmacy is intended to supply a certain population in medicinal products, magistral medicines and other products catalogued in the Art. 86 (8) in conjunction with the Art. 72 (5) of the Ph. Law Act.

On the basis of the above-mentioned, pharmaceutical services include dispense of medicinal products and medical devices, preparation of magistral medicines and information to be provided about the medicinal products and medical devices (Art. 86 (2)). Pharmacy therefore under the Ph. Law Act is not equated with a place of drug sales but a place of drug dispensing. On the other hand, reimbursement margin pertains to a concrete drug or medicinal product not to the pharmaceutical service. Non-reimbursement margin does not include pharmaceutical service as well, which, to be mentioned, is not valuated at all. The cost of pharmaceutical service is not comprised into the drug price except magistral medicines, which are prepared based on the patients’ prescription. Therefore, pricing of a magistral medicine include the so called “taxa laborum” understood as the cost of this customization (2).

In theory, the pharmaceutical sector may be identified by two extreme market models: central, in which the whole market is managed and controlled by the governmental institutions and model based on the market mechanism, which functions basing on a competition of a wide range of manufacturers and buyers. Polish pharmaceutical market is an example of model in which some factors of a central model enact together with those classified to the market mechanism. Therefore, a community pharmacy in Polish system of health care is to be placed in a very distinctive way. Retail sale is carried mostly under the market-driven rules – what can be illustrated by demonopolization and a possibility of running a pharmacy by a various entities, non-necessary with a pharmaceutical diploma and the freedom of choosing a form of this business activity. At the same
time, pharmacy is a subject of certain limitations, for example, the number density ban or legally obliged to work in defined hours and heavily controlled by the governmental institutions (2). Although such scenario is present in many countries, where governments do apply limits for the market mechanism in health care, some of those countries also additionally support this specific sector for example by guidelines aiming at patient security, expenditures rationalization and minimization of misunderstanding the applied legal rules.

According to IMS Health, at the end of the year 2015 there were 14569 pharmacies in Poland, out of which 5400 operating in chains. Analyzing the demographical data, the average pharmacy in Poland covers 2734 citizens, in comparison Germany 3958, Spain 2203, the United Kingdom 4452, The Netherlands 8467 and Czech Republic 4351. In the year 2013 in the pharmacists’ opinion, as the IMS Health claims, 52% of pharmacies highlighted the fallout of financial situation, moreover, 21% fallout of ability to pay its suppliers on time. As key points threatening the pharmacies situation in the following year the rising market share of pharmacies in chains, unclear, incoherent law and competition were indicated. The same source points out that 78% of closed pharmacies in early 2014 were the individual ones. Almost 30% of pharmacies are branch pharmacies, which in fact, generate over 44% of market sales – such trend shall continue to grow (3). IMS Health indicates that pharmacies in middle branches (15-29 and 30-49) operate most effectively – meaning high sales, low prices and efficient inventory turnover.

Branded-to-generic, generic-to-generic and generic-to-branded substitution

Substitution is a process of switching the prescribed pharmaceutical product into its available equivalent. Such process arises in pharmacy between a pharmacist or a pharmacy technician and a patient, with an indirect participation of a physician and, in terms of reimbursement pharmaceuticals, commonly a public payer. Substitution occurs both in branded (branded product understood by Authors as innovative, reference product) and generic scope. A generic drug, as World Health Organization (WHO) claims, is a pharmaceutical product in principal intended to be switched, what must be highlighted, with an innovative product, launched after innovatives’ patent expire. WHO recommends trade of generics to be carried out by an international nonproprietary name (INN) rather than a proper name (4). The Art. 15 of the Ph. Law Act defines the equivalent (so the generic) drug as a product with the same qualitative and quantitative composition, the same pharmaceutical form as the reference drug, and which bioequivalence with the reference product has been confirmed by the bioavailability tests. Salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of an authorized active substance are to be the same active substance unless differ remarkably from this active substance with its characteristics in terms of safety or efficacy. If so, the marketing authorization holder encloses documentation confirming the safety or efficacy of salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of an authorized active substance. Reference product (the innovative), unlike the generic, is authorized after pre-clinical then clinical trials. Generic drugs are subject only of bioequivalence verification with the reference product. Therefore, it is stated that a similar profile of biologically active compound changes in blood a base to assume that both drugs indicate similar efficacy and safety. The rules of bioequivalence tests on the European market arise from the European Medicines Agency (EMA) guidelines. It is stated that bioequivalence tests are carried out on healthy volunteers. Each volunteer obtains both innovative and generic drug usually in a random order (5). Permissible percentage of absorbed dosage, max blood concentration level and time after which it will appear may differ. Commonly practiced range of biological equivalence is between 80%-125%. The Orange Book – Approved Drug Products with Therapeutic Equivalence Evaluations issued by the Food and Drug Administration (6) – FDA divides medicines into two categories – A, which classifies therapeutic equivalent medicines with the reference ones and B, which classifies medicines not therapeutically equivalent with the reference medicines, due to differences in bioequivalence, which most commonly arise from specific pharmaceutical form or specific pharmacokinetics (7). Going further, in category A there are two subcategories – first, in which biological equivalence is not an issue, and second in which biological equivalence with the reference drug is confirmed by the in vivo tests. In the U.S. FDA is responsible for supervision of drug registration and directly points which drug is a reference one in the Reference Listed Drug (RLD) list. Accordingly, consecutive generic products being the subject of an authorization (by Abbreviated New Drug Application, ANDA) refer to the same reference product (6). Furthermore, only reference drug occurs under the brand, registered name, generics under generic names – INN, as
WHO recommends. Polish factual data differ from above-mentioned model. Guideline on the Investigation of Bioequivalence, CPMP/EWP/QWP/1401/98 Rev. 1 (European Medicines Agency) contains two criteria, which determine the rules of choosing a reference product in respect to the generic product being authorized on the European market – those are actual register in the European Union and marketing authorization based on the full documentation agreeable with the Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use. The content of active substance in series of tested and reference product may vary maximum by 5%. What is meant by above, the selection of a reference product lies on the applicant unlike the U.S. market, where such product is issued by an marketing authorization holder.

Reimbursement policy
Current pharmaceutical law (not only in Poland) indicates tendency for applying substitution/switching solutions due to rational savings in health care system. There are scientific proves of “prosubstitution” solutions under the pharmaceutical law, which aim to increase the share of generic drugs on the reimbursement drug market (8, 9). For substitution to arise legally in Poland, the following criteria must be fulfilled (according to Art. 44 of the law of 12 May 2011 on the reimbursement of medicines, food products of special nutritional purpose and medical devices – Reimb. Act):
1. Both pharmaceutical products must have the same international name, dose and pharmaceutical form, which does not give rise to therapeutic differences and has the same therapeutic indication;
2. Retail price of which does not exceed the public fund financing limit;
3. A pharmacy is obliged to ensure that such medicine is available (10).

The criteria mentioned above concern also medical devices and foodstuffs for particular nutritional uses. It is possible to switch medicine not only to less expensive one but, since summer 2016, also to the one having the same price, more expensive drug or a drug, which is not reimbursed – 100% patient surcharge (11). Such act of change is to be a top-down step forward to the sustainable development and ease to an individual approach to a patient undergoing a substitution process. Situations (for patient) of not receiving a prescribed drug, which was the cheapest one in a single limit group, because of its lack in retail and wholesale sale are, prospectively, no longer to appear. Since autumn 2016,

<table>
<thead>
<tr>
<th>Limit group 204.0</th>
<th>Leukotriene receptor antagonists - other oral drugs used in obstructive pulmonary diseases (LTRAe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. Pr.</td>
<td>20.09</td>
</tr>
<tr>
<td>Nom. Pr.</td>
<td>11.08</td>
</tr>
<tr>
<td>3</td>
<td>14.04</td>
</tr>
<tr>
<td>4</td>
<td>15.12</td>
</tr>
<tr>
<td>5</td>
<td>22.57</td>
</tr>
<tr>
<td>6</td>
<td>15.30</td>
</tr>
<tr>
<td>7</td>
<td>14.04</td>
</tr>
<tr>
<td>8</td>
<td>11.23</td>
</tr>
<tr>
<td>9</td>
<td>20.52</td>
</tr>
<tr>
<td>10</td>
<td>16.74</td>
</tr>
<tr>
<td>11</td>
<td>5.81</td>
</tr>
<tr>
<td>12</td>
<td>10.15</td>
</tr>
<tr>
<td>13</td>
<td>17.88</td>
</tr>
<tr>
<td>14</td>
<td>38.66</td>
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<tr>
<td>15</td>
<td>22.14</td>
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<td>16</td>
<td>12.31</td>
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<td>17</td>
<td>15.12</td>
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<tr>
<td>18</td>
<td>10.80</td>
</tr>
<tr>
<td>19</td>
<td>14.04</td>
</tr>
<tr>
<td>20</td>
<td>21.58</td>
</tr>
<tr>
<td>21</td>
<td>7.13</td>
</tr>
<tr>
<td>22</td>
<td>8.91</td>
</tr>
<tr>
<td>23</td>
<td>17.82</td>
</tr>
<tr>
<td>24</td>
<td>17.82</td>
</tr>
<tr>
<td>25</td>
<td>7.56</td>
</tr>
<tr>
<td>26</td>
<td>9.72</td>
</tr>
</tbody>
</table>

Figure 1. Example of a single limit group calculation (in PLN)
patients over 75 years old are to receive medicines free of charge (12).

Under the Polish law, it is absolutely intolera-ble to perform therapeutic substitution, understood as switching medicines with similar therapeutic effect but with different active ingredient. It is also absolutely intolerable to switch medicine, which has been prescribed by an authorized person with the appropriate note “dispense as written”. Under the Reimb. Act (Art. 44) one and only possibility of dispensing equivalent of prescribed medicine is evident and categorical patients’ claim after being informed by a pharmacist/technician about available equivalent. Setting prices and margins for the reimbursed medicines are strictly defined in the Reimb. Act. At first, manufacturers negotiate prices with the Ministry of Health (official price) – a new reimbursement list is launched every two months (however, a drug is placed on a reimbursement list for at least two years). Wholesalers’ margin is set on the level of 5% and is calculated from the official price. Retail margin is calculated from the wholesalers’ price of a medicine being a base in a certain limit group as stated in the Art. 7 (4) of the Reimb. Act (see Fig. 1).

According to the Reimb. Act (Art. 14) there are four payment categories of a reimbursed drug: free dispensing, flat-rate, 30% and 50%. All those entitle to a public financial limit and with eventual patient surcharge as an effect of a difference between retail price and the level of a public financial limit. Public financial limit is directly colligated with a price of a medicine, foodstuffs intended for particular nutritional uses and medical devices, which is a limit base in each limit group. The amount of pay differential between the official retail price and the publicly financed sum of a certain medicine is a patient’s surcharge (Fig. 1). In consequence, for example, for a medicine with free dispensing it is not univocal that a patient will receive it without any charges.

DISCUSSION

Law and economics, as defined by Posner, is the “application of the theories and empirical methods of economics to the central institutions of the legal system” (13). Authors do take issue with the far-reaching interpretations that efficiency should be the goal of law and do agree with the Hanson’s conclusions defining the field as understanding the law effects itself and how those laws might be altered to better serve the goal of efficiency (13). Efficiency itself aims at minimizing the waste of resources, including energy, time and materials while successfully completing the desired gain. System of health care faces different, sometimes contrary challenges – a well-being of a patient from one hand, profits for the commercial entities on the other hand, including pharmacies and sources for maintaining its workers. For a necessary harmonization it is needed to work for solutions, which do protect patients but also do not conflict in business activities of entities delivering health care services to patients. In terms of pharmacy, facing competition may go hand in hand with its social, even servant profile. Globally inevitable phenomenon of pharmacies connecting into chains raises a question: does a purely economic understanding of effectiveness of chain pharmacies has a free space for efficient fulfillment of public health protection acts (for example by a pharmaceutical service). Does market acts and competition resulting from legal capacity of a pharmacy, which in fact, is an enterprise, shall give way to its social, even servant obligations. Therefore, should an issue of pharmacy operating effectively be widen to its superior purpose. Does the problem of market manipulation, understood as the “utilization of cognitive biases to influence peoples’ perceptions and, in turn, behavior” is to be acknowledged as possible in current reality (14). Pharmaceutical services need to be distinguished from the health services, which are acts aiming at health remaining, saving, recover or improvement and other medical acts arising from the treatment process or other regulations defining the rules of its execution (12). Pharmaceutical service is, without a doubt, a service, which aims at remaining, saving, recovering or improvement of health but it is a subject undefined and not included in any legal act as the health service.

The acknowledgement of a drug to be bioequiv-alent does not guarantee therapeutic equivalence to the reference drug. Therefore, it is a subject of a constant discussion, does proven bioequivalence is a sufficient argument to substitute pharmaceutical products or maybe it should be determined by the clinical trials, which result whether a generic drug is therapeutically equivalent with an innovative one (7). It should be highlighted that bioequivalence test is performed always in comparison with the reference product. Those tests are not held between two generic products, therefore the substitution, which takes place in a pharmacy, between two generic products is not confirmed by any knowledge of their bioequivalence towards each other. Each of those generics could differ by 80%-125% from the reference drug. Therefore, it is hard to confirm the exact difference between two generic pharmaceutical products.
The availability of generic drugs on the market drives the competition, mostly between pharmaceutical companies, which in the end, magnifies the accessibility of low-priced offered medicines to patients and public payer. Generic products dominate the pharmaceutical market in Poland. In terms of sales share by volume they stand for 84% of total and, in terms of value 62% (year 2014) (15). According to Deloitte, the U. S. generic drugs account for around 70% of the U. S. drug market by volume. In Europe around 50%, however the proportion differs significantly by country (16). What is interesting, Deloitte highlights “in the U. S. generic use is almost 90% within the off-patent market but in many European countries potential savings are not fully exploited due to lower utilization of generics in key therapy areas”. As already has been indicated in the literature (5, 17-19) in terms of narrow therapeutic index (NTI) drugs “routine switching between different manufacturers of antiepileptic drugs should be avoided”.

In Polish factual data substitution does not mean switching only to a generic product. Some original pharmaceuticals, which are not a subject of reimbursement, are cheaper than their reimbursed equivalents. In factual data, substitution arises both in reimbursed and payable in full medicines.

In factual data a pharmacy as an entity, in the beginning is obliged to buy certain medicines, apply certain retail margins and when a patient arrives with a certain prescription, for example of free dispensing, dispense this medicine according to its category. Under the Reimbursement Act twice a month a pharmacy is obliged to send to a public payer (National Health Fund) reports about reimbursement it has made during 14-day-period and only then will receive invested earlier money (see Fig. 2). The INN prescribing is of high matter for the harmonic substitution process (see Fig. 3). A top-down approach and number of reforms, including physician incentives, are needed to successfully change prescribing habits. INN prescribing increases the role of pharmacist, who can then perform a pharmaceutical service to best suit individual a medicinal product, and do not conflict with the pharmacy as a business entity. Frequent changes in reimbursement list in Polish setting – every two months – make an inventory turnover difficult to perform effectively. In France for example, ambulatory care physicians agreed to prescribe by INN in return for higher fees (20).

A tendency of legal authorities aiming at looking for cost savings seems likely to lead to an increased use of generic drugs in key therapy areas, where it is safe to occur. A priori substitution process leads to cost savings. Therefore, it is important to support pharmacies with substantial legal “tools” of such process in various therapeutic areas (different for example cardiovasulars and AEDs) symbiotic with pharmacy being a business entity.

**Figure 2. Example of a possible scenarios of receiving a margin (in PLN) for a medicine ketoprofen 1 in accordance with the Reimbursement of Medicines, Foodstuffs Intended for Particular Nutritional Uses and Medical Devices law of 12 May 2011**
Each person undergoing a substitution should be assessed carefully. An electronic view of medical history of a patient, where a pharmacist may see what medicines this particular patient is currently taking and which has already been substituted in the past, may eliminate potential mistakes of not adequately enacted (due to low information provided) process of substitution. The U. S. has provided its medical professionals with the Orange Book and clearly defined processes of a drug being the reference one, therefore the U. S. market may benefit from the savings it has made during a decade – $1.2 trillion (60% of savings in nervous and cardiovascular systems) (21).

It seems likely that the next few years will undergo a wider discussion on systemic sustainable development of a substitution process from the beginning, so medicine being registered till its dispensing and control of an enacted process. As it may be the INN prescribing, as in the United Kingdom, will be raised on the merits. Percentage of INN prescribing is the highest (over 80%) in the UK, the USA and China, which, growing fast, is an interesting example – its Government ensured 90% health coverage for its citizens (2009-2011) (22).

CONCLUSIONS

Nontransparent drug registering methods, nontransparent side effects monitoring (as well as access to such base), frequent changes in reimbursement drug list and lack of cooperation between various medical professions (ex. lack of access to patients’ taken and prescribed drugs for pharmacists) stay in conflict with the harmonic (for all stakeholders) process of drug substitution. Relevance of registering method of a generic drug is of high matter for the substitution process. Substitution, under law, should be held in accordance with the therapeutic areas in order to fully exploit benefits of such process. Law has its other goals than efficiency, however, it can be altered to better serve the goal of efficiency of a system as a whole. Pharmacy is a place of public health protection – each country, which in fact, is in disposal of the instrument such as law, may control both expenditure rationalization of members of health care system and provide them with essential, needful tools for sustainable development. Legislator, by including in pharmacy definition words “public health protection” on one hand obligates this entity to fulfill its crucial, overarching goal from the other, therefore, leaves to the market self-law. The INN prescribing should be legally introduced as compulsory. As evaluated by Deloitte “there is a strong trend towards consolidation and globalization of legacy systems into single, global drug safety system with harmonized business processes”. Authors do believe that such trend will reach Poland visibly in upcoming years.
REFERENCES


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Non-conventional formulations such as pharmaceutical drug delivery systems containing a delivery base and an active agent have created new application rules for pharmacist and patients in therapies (1). Although the patient’s adherence related to the application of medicinal patches (Transdermal Therapeutic Systems) is relatively high compared to the other dosage forms (2, 3), the lack of patients’ knowledge about the correct use of medicinal patches could decrease the effectiveness of the therapy. The knowledge of standard application rules related to this non-conventional dosage form is essential belonging the maximum effectiveness of therapy (1, 4, 5).

Health literacy concerns the knowledge and competences of persons to meet the complex demands of health in modern society (e.g., personal communication skills or reading comprehension skill (6)).

The health literacy and the pharmaceutical counseling (e.g., patient education with written information) are closely related in community pharmacy (7). The evaluation of health literacy can contribute to the development of the adequate counseling and services as well as in the necessary pharmaceutical therapeutic interventions of different groups of patients (8). The pharmacists have to recognize the lack of health literacy in community pharmacies and consequently to improve the patient’s knowledge and comprehension with effective practice. There are several international formal assessments of health literacy (9, 10).

The aim of the patient education is to increase the patient’s adherence and the effectiveness of drug therapy (11) with adequate and practical applications in general practice settings (12), therefore the barriers or facilitating factors and the health outcomes should be determined according to health literacy (13).

Special health literacy studies were developed by researchers related to different therapies, dosages forms or the different types of patients’ information

THE EFFICACY OF WRITTEN INFORMATION ABOUT THE APPLICATION RULES OF TRANSDERMAL PATCHES – READING COMPREHENSION QUESTIONNAIRE SURVEY IN HUNGARIAN COMMUNITY PHARMACIES

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Abstract: Although patient’s adherence related to the application of transdermal patches is relatively high compared to the other dosage forms, the lack of correct use could decrease the effectiveness of the therapy. Therefore, the primary aim of our study was to evaluate the efficacy of a Patient Information Leaflet (PIL) concerning the adequate use of medicinal patches. The questionnaire survey was developed to explore the level of patients’ reading comprehension in four Hungarian community pharmacies. The distribution of four separated comprehension categories was measured. For the hypothesis testing it was assumed that the reading comprehension of “patch ever adopters” is higher than “patch never adopters”.

46% of participants (n = 163) has “adequate”, 40% has “high sufficient”, 12% has “low sufficient” and 2% has “inadequate” level of reading comprehension based on responses. The hypothesis testing showed no significant difference (p = 0.428). The instructions of PIL are effective although the majority of participants (54%) has lack of complete understanding. Patients have not met these application rules, according to hypothesis testing. Written medicine information is useful in patient education, but PILs cannot replace the pharmacists’ obligation to provide verbal counseling.

Keywords: transdermal patch, counseling, community pharmacy, written information, reading comprehension, questionnaire survey

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(e.g., written, verbal or online information). The measuring of reading comprehension helps to evaluate the efficacy of written information (e.g., texts of Patient Information Leaflets – PILs) and enables the development of understandable text for patients (with illustrations) in the serious therapeutic issues and in different patient’s groups (14-22).

Short, concise messages (consumers prefer these e.g., in “bubble format”) (20) should be created with familiar words and recognizable icons (23). Beside the manufacturers’ leaflets concerning the application rules of medicines, patients receive useful written information about the new medications. Although the patients can read the instructions, but the leaflets should not replace the pharmacist’s obligation to provide verbal counseling in pharmacies (24).

Pharmacists and the community pharmacies’ staff need additional training regarding health literacy and new services should be developed in focus of patients’ knowledge in order to enhance the effectiveness of home medicine use (e.g., easy-to-read printed materials in pharmacy) (25-28).

The primary aim of the present study was to evaluate the efficacy of a self-developed Patient Information Leaflet (PIL) concerning the adequate use of transdermal patches and the consequent pharmacists’ intervention in Hungarian community pharmacies in order to improve the patients’ knowledge about the correct application.

EXPERIMENTAL

The survey was administered in four community pharmacies in two different regions of Hungary (Central Hungary and West Hungary) from March of 2016 to April of 2016. The pharma-

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Table 1. Tasks of reading comprehension test, the type of the questions and the corresponding scoring system of assessment.

<table>
<thead>
<tr>
<th>Reading comprehension tasks</th>
<th>Type of question</th>
<th>Correct answer or valuable answers</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>The small lesions on your skin surface might not affect the absorption of the drug, just the visible bigger lesions</td>
<td>True - false</td>
<td>False</td>
<td>1</td>
</tr>
<tr>
<td>Each patch can be cut to modify the dose of the active agent</td>
<td>True - false</td>
<td>False</td>
<td>1</td>
</tr>
<tr>
<td>The well-selected place of patches is important on your skin for the adequate protection and tight adhesion and it contributes to the systematic exchange of patches</td>
<td>True - false</td>
<td>True</td>
<td>1</td>
</tr>
<tr>
<td>If you would like to use a patch on the same skin area again, you must let the affected surface be patch-free for at least 1 day</td>
<td>True - false</td>
<td>False</td>
<td>1</td>
</tr>
<tr>
<td>So-called surfactants chemicals for example in soap, in oils and in creams can modify the transdermal absorption of the active substance</td>
<td>True - false</td>
<td>True</td>
<td>1</td>
</tr>
<tr>
<td>Circle the letter of the correct applications (You can mark more than one answer)</td>
<td>Multiple choice</td>
<td>Only d) and e) marked</td>
<td>3</td>
</tr>
<tr>
<td>a) Before applying a medicinal patch you should clean the surface of skin with soap.</td>
<td>d) and e) + other marked answer (s)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>b) Before applying a medicinal patch you may not clean the surface of skin.</td>
<td>Only d) or only e)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>c) Although it is necessary for reason of bad adhesion, depilation is not allowed at the affected skin before applying a medicinal patch.</td>
<td>Only d) + other marked answer(s) or only e) + other marked answer(s)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>d) It is not allowed to use a razor for the affected skin depilation before applying a medicinal patch.</td>
<td>Not marked d) and e)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>e) After sticking you should press the transdermal patch to the skin and you can fix it in another way (for example with other adhesive-tape), if it is necessary.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f) The best decision is a medicinal patch dosage form for the local treatment of small joint’s pain.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The efficacy of written information about the application rules of... 1609

acies were a varied of low, medium and high traffic community pharmacies. The regular procedure was ensured by staff of pharmacies (pharmacists and assistants). As each patient voluntarily participated in the survey, men and women over 18 years of age answered the questions on paper in pharmacies. Answering the questionnaire took about fifteen to twenty minutes.

At first, the text of PIL was developed based on evidence (1, 4, 5) and counseling guidelines of “health literacy friendly pharmacies” (29). The original text was written in Hungarian avoiding the technical words (using familiar words), the most important instructions were in bullet points and the keywords were highlighted in the text. The size of paper with text was “A5” or “A4” for better readability who did not see the small font size. The Appendix is the English version of the self-developed PIL concerning the adequate use of transdermal patches.

The questionnaire survey basically organized from three parts and one cover letter with introduction and participation information, as follows:
1. page: the cover letter with introduction and information for participants;
2. page: questions regarding demography and personal use of medicinal patches (most of the questions are closed-type);
3. page: the developed text of PIL concerning the most important application rules of transdermal patches;
4. page: reading comprehension test and a question concerning the additional request of the patients (only closed-type questions).

In the course of the answering of the part of reading comprehension test it was allowed to use the PIL without personal assistance of the staff.

The reading comprehension test consisted of five true / false type questions and a multiple choice task. The last question explored to public opinion about demand of verbal counseling related to correct use of non-conventional drug formulations.

Table 1 summarizes the content and type of reading comprehension tasks and the scoring system of assessment.

Four reading comprehension levels have been separated with an eight-point scale based on the maximum available eight points. The four categories are as follows:
• 0 – 2 points: “inadequate” level of reading comprehension;
• 3 – 4 points: “low sufficient” level of reading comprehension;
• 5 – 6 points: “high sufficient” level of reading comprehension;
• 7 – 8 points: “adequate” level of reading comprehension.

Statistical evaluation of the results

IBM SPSS statistics was applied for the evaluation of the results. For hypothesis testing and the more other descriptive statistical tests χ² tests were selected. Kruskall Wallis test was used for age groups analysis in eight-point scale without separated comprehension categories.

For the hypothesis testing it was assumed that the reading comprehension of “patch ever adopters” is higher than “patch never adopters” because “patch ever adopters” have known the corresponding application rules.

RESULTS

163 patients (113 women and 50 men) with the age distribution represented in Table 2 participated in the survey. Based on the responses 82 participants have been used transdermal patch at least once, 10 participants are applying a patch at the time of survey and 71 participants have never used medicinal patch.

According to indications most of the participants (75 persons) have used the transdermal patch for pain and inflammation relief (NSAIDs content – OTC) and in cases of chronic therapies most of the

<table>
<thead>
<tr>
<th>Reading comprehension</th>
<th>Age (years)</th>
<th>Frequency (persons)</th>
<th>Mean Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18-30</td>
<td>29</td>
<td>89.60</td>
</tr>
<tr>
<td></td>
<td>31-50</td>
<td>61</td>
<td>88.89</td>
</tr>
<tr>
<td></td>
<td>51-70</td>
<td>58</td>
<td>73.53</td>
</tr>
<tr>
<td></td>
<td>70-</td>
<td>13</td>
<td>58.12</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>161*</td>
<td></td>
</tr>
</tbody>
</table>

*missing dates are in two questionnaires
patients (6 persons) have used this dosage form for pain relief of cancer diseases (fentanyl content).

74% of “patch ever adopters” (92 persons) could name one product at least (the brand-name of patch was well written) related to the selected indication of their therapy.

The results of the measured reading comprehension is “adequate” (7–8 points) in 46% of 163 participants, 40% has “high sufficient” (5–6 points), 12% has “low sufficient” (3–4 points) and 2 percent has “inadequate” (0–2 points) level of reading comprehension.

The hypothesis testing did not show a significant difference between the reading comprehension of two groups related to transdermal patch use (p = 0.428 with 95% confidence intervals).

The difference was significant, according to difficulty of reading comprehension tasks: p < 0.001 and $\chi^2 = 87.519$ in six questions. The multiple choice task was the most difficult for patients; in this case 37% of participants could answer without error (3 points).

The difference of reading comprehension is nearly significant by age (p = 0.051). Table 2 illustrates the results of Kruskall Wallis test. The results indicate that the reading comprehension of 18–50 year-old participants is better than the group of aged 51 and over in practice.

The responses of the special request concerning the pharmacists’ information service show the demand of personal pharmaceutical counseling related to non-conventional dosage forms in community pharmacies. 80% would require this service, 14% selected the “I don’t know” choice and 6% would not require advices from pharmacist.

**DISCUSSION AND CONCLUSION**

The self-developed PIL has been effective concerning the correct use of transdermal medicinal patches because in most of cases (86%) the level of reading comprehension was “adequate” (46%) or “high sufficient” (40%).

The authors’ previous questionnaire survey was developed to explore the level of patients’ knowledge of the correct use of transdermal patches without counseling based on their own applying habits. It was administered in thirteen Hungarian community pharmacies from October of 2012 to May of 2015. Most of the participants, including men and women over 18 years of age (n = 233), used major analgesic patches (fentanyl); the remainder were given nitroglycerin, NSAID analgesics patches during the survey. Based on patients’ applying habits only 9 tests were flawless from 233 completed questionnaires (30).

Results of reading comprehension study and our previous survey confirm that the text of PIL is sufficiently readable and understandable for the improvement of the patients’ knowledge related to use of transdermal patches at home. A similar American survey was determined to evaluate whether patients read non-manufacturer-developed leaflets and assess patients’ opinions concerning the understandability and usefulness of these leaflets (24). Approximately two-thirds of the patients surveyed reported that they least frequently read the leaflets provided with new medications. The majority (over 90%) reported the leaflets to be useful and easy to understand (in our study it was measured: 86%). Pharmacists should advocate reading the leaflet and promote it as a useful resource (24).

Although the written instructions have proved useful, 54% of participants has lack of proper understanding (“high / low sufficient or inadequate” level of reading comprehension), the PIL should not replace the verbal counseling and patient education related to this dosage form. This finding is similar to the result of a study concerning hormone therapies. The assessment of the readability of written information of 31 hormone therapy products showed that the majority of the materials are written at a high reading level. These findings have implications for health literacy and counseling efforts when helping women to understand their options for menopausal symptom management (15). Pharmacists may need to supplement written information with additional, more understandable self-developed PILs or “patient-friendly” verbal instructions (15).

There is a definite demand (based on the requests of 80% of respondents) for verbal counseling concerning the application rules of non-conventional dosage forms mainly in cases of complex instructions. The multiple choices were the most difficult task in reading comprehension test (only 37% of responses were correct) opposite the true / false type questions. Usually complex information caused difficulties for patients, e.g., according to another study, patients of all health-literacy levels had better understanding of medication warning labels that contained single-step versus multiple-step instructions in primary care clinic at the Louisiana State University (23).

The hypothesis testing showed no significant difference between reading comprehension of “patch ever adopters” and “patch never adopters”.

The latter demonstrates that the patients were not familiar with these practical application rules of transdermal patches. In community pharmacies the
pharmacists should inform patients about the importance of correct home medicine use. The self-written PIL, along with the verbal counseling can effectively contribute to the successful drug therapy.

The self-developed PIL is useful to give the transdermal patch adopters in community pharmacies along with the point by point verbal explanations. The pharmacist should pay special attention to patients over 50 years of age in order to reach an effective and safe home use of transdermal patches.

Finally, the pharmacists have essential role in community pharmacies to improve the health-literacy competencies of consumers or patients in general. Although the majority of consumers had adequate skills, which was confirmed by several international studies, in specified cases (e.g., complex information, advanced age, less formal education) additional written and verbal information interventions are required by pharmacy staff.

Acknowledgments

The authors are grateful to the Patika 52 Pharmacy, Patika Libra Pharmacy, Kiscelli Pharmacy, Celli Szent Márton Pharmacy and the Institute of Behavioral Sciences Semmelweis University for their valuable contribution in the survey.

Ethical approval

Not required. Each pharmacy signed a declaration confirming the regular procedure. As each patient voluntarily participated and was accordingly informed in the survey, which is a non-interventional study, therefore no further ethical approval was required.

Funding

None.

Competing interests

None declared.

REFERENCES

Appendix

Patient Information Leaflet concerning the correct application rules of transdermal patches

Please, read the text concerning the application rules of medicinal patches.

✓ Before applying a medicinal patch you should never use a razor for skin depilation because a razor can cause small (micro) lesions on your skin’s surface, which might affect the absorption of the drug. If necessary, use scissors for skin depilation (1)!

✓ The patch should be applied to clean, dry skin. You may cleanse the skin but use only clean water and then wipe the skin, because any chemicals (for example soap) can modify the liberation and absorption of the active substance from the patch (1, 4).

✓ Avoid using oils and creams before applying the patch because they too can damage the effect of the drug and the adhesion of the patch (1, 4).

✓ The location of patch application should be alternated. If you would like to use a patch on the same skin area again, you must let the affected surface be patch-free for at least 3 days (1, 4).

✓ In chronic illness therapies where the aim is the effects on the whole body (not one body part’s pain and inflammation relief) it is necessary to selected a protected surface of skin from external effects but it is not necessary to place the patch in the middle of your back because it is difficult to affix. You should look for a place on your body where you can see the patch well and do not forget to change the location, for example, shoulders, scapula, abdominal wall (1, 4, 5) etc.

✓ In applying a patch, ensure that it adheres tightly to the surface of your skin. Avoid the body’s bights or small joints (knees, wrist, underarm, elbows, etc.). The adequate dosage forms are the gels or creams for the treatment of these bights or small joints, not the medicinal patch. In applying exert pressure on the patch on your skin for 30 s. If necessary, use another adhesive-tape to fix the medicinal patch (1, 4, 5)!

✓ Always read the patient information leaflet of manufacturers primarily related to information about cutting. Although cutting can cause damage of the structure of some patches, you can cut other patches to modify the dose of the active (for example sometimes it is allowed to cut in half) (4, 5).
Health screening programs in community pharmacies and their importance of identifying people with chronic diseases such as diabetes or hypertension are well known. The results of these services could initiate appropriate treatment and prevent long-term complications from chronic diseases. Diabetes mellitus and hypertension are major global problems. The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014. Data from 2015 indicate that one in 11 adults has diabetes. In 2040, about one in 10 adults will be sick. Moreover, one in two adults has undiagnosed diabetes (1). Globally, the overall prevalence of increased blood pressure in adults aged 25 and over was about 40.0% in 2008. The number of people with uncontrolled hypertension increased from 600 million in 1980 to nearly 1 billion in 2008 (2). This dramatic numbers brought about many changes and activities in health promotion around the world.

It is very important to perform health screenings in each population. Community pharmacies in many countries have implemented screening services which are very sensitive and effective. In the Swedish study, 6.9% elderly persons in community pharmacies in each country were screened for diabetes and hypertension.

Abstract: The prevalence of chronic diseases increases in the society. The health care system should be focused on improved disease prevention and health promotion. The pharmacist is very often the first health professional contacted by the patient. It would be beneficial for society to provide health screening services in a community pharmacy. The aim of the study was to evaluate and compare opinions about health screenings for chronic diseases in community pharmacies located in Poznań, Poland, and Chicago, USA. An anonymous simple 12-item questionnaire was developed in Polish and English to assess attitudes about screening services in a community pharmacy. The questionnaire as a cross sectional study was carried out from March 2016 to June 2016 within patients in community pharmacies in Poznan (n = 265, 37.0% men and 63.0% women) and in Chicago (n = 190, 42.6% men and 57.4% women). A majority of respondents, 72.1% in Poznan and 77.0% in Chicago, confirmed that information about prevention of common chronic diseases was expected to be provided in a community pharmacy. Both groups were also interested in participation in health screenings for common chronic diseases in a pharmacy (66.8% Poles and 58.0% Americans). Respondents in Poznan prefer to have such services paid by National Health Fund while in Chicago, by a community pharmacy (p < 0.001). These findings show general interest in participating in health screening services provided by pharmacists. Development of new screening services in community pharmacies may help to prevent or lower the risk of complications associated with common chronic diseases.

Keywords: screening services, health promotion, community pharmacy, pharmacist, chronic diseases
pharmacy were detected with suspicions for diabetes type 2 and 71.5% had at least two risks factors of this disease. In addition, 54.0% individuals showed elevated blood pressure and 16.3% had hypertension (3). The Thai Diabetes Prevention Program in community pharmacies identified that half of the tested clients were at risk of diabetes and provided an opportunity for participants to learn about the prevention of diabetes. During a 3-month service for 397 individuals, nearly half were at a high risk for diabetes (4). These studies showed that it is very important to implement a screening campaign for chronic diseases in a community pharmacy. A sequential screening in pharmacy practice may detect up to 7% patient suspected for diabetes. Most people visiting community pharmacy are open to counselling about lifestyle. Health screenings are recommended in asymptomatic adults, including those with smoking habits, high blood pressure, high cholesterol, and obesity (5).

Pharmacists working in community pharmacies are respected and trusted by society and therefore are in an excellent position to engage in screening, monitoring and educating patients with potential problem of chronic diseases. Community-based screening programs can result in delay of complications of chronic diseases and improved quality of life.

The aim of the study was to evaluate and compare opinions about health screening services in community pharmacies located in Poznań, Poland, and Chicago, USA. The effect of age, gender and education was evaluated.

**MATERIALS AND METHODS**

The survey as a cross sectional study was carried out from March 2016 to June 2016 in community pharmacies. It covered 265 respondents in Poznań (37.0% men and 63.0% women) and 190 participants in Chicago (42.6% men and 57.4% women) selected at random. They were patients of independent community pharmacies in Poznań and Chicago that voluntarily agreed to fill out the questionnaire. The most numerous group of respondents consisted of Poles aged from 18 to 30 years old (32.4%) and Americans aged from 18 to 30 years old and from 41 to 50 years old (in both cases 23.2%). The majority of the responding participants in Poznań and Chicago had graduate education (37.7% and 37.9%, respectively). Socio-economic data included information about gender, age and education.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Poznań n (%)</th>
<th>Chicago n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>167 (63.0)</td>
<td>109 (57.4)</td>
</tr>
<tr>
<td>Male</td>
<td>98 (37.0)</td>
<td>81 (42.6)</td>
</tr>
<tr>
<td>Total</td>
<td>265 (100.0)</td>
<td>190 (100.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age(years)</th>
<th>Poznań n (%)</th>
<th>Chicago n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-30</td>
<td>86 (32.4)</td>
<td>44 (23.2)</td>
</tr>
<tr>
<td>31-40</td>
<td>34 (12.8)</td>
<td>36 (18.9)</td>
</tr>
<tr>
<td>41-50</td>
<td>60 (22.6)</td>
<td>44 (23.2)</td>
</tr>
<tr>
<td>51-60</td>
<td>55 (20.8)</td>
<td>29 (15.3)</td>
</tr>
<tr>
<td>61-70</td>
<td>19 (7.2)</td>
<td>28 (14.7)</td>
</tr>
<tr>
<td>&gt; 70</td>
<td>11 (4.2)</td>
<td>9 (4.7)</td>
</tr>
<tr>
<td>Total</td>
<td>265 (100.0)</td>
<td>190 (100.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Education</th>
<th>Poznań n (%)</th>
<th>Chicago n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>4 (1.5)</td>
<td>3 (1.6)</td>
</tr>
<tr>
<td>Vocational</td>
<td>32 (12.1)</td>
<td>17 (8.9)</td>
</tr>
<tr>
<td>High school</td>
<td>62 (23.4)</td>
<td>58 (30.5)</td>
</tr>
<tr>
<td>College</td>
<td>67 (25.3)</td>
<td>40 (21.1)</td>
</tr>
<tr>
<td>Graduate</td>
<td>100 (37.7)</td>
<td>72 (37.9)</td>
</tr>
<tr>
<td>Total</td>
<td>265 (100.0)</td>
<td>190 (100.0)</td>
</tr>
</tbody>
</table>
The study confirmed that higher percentage of respondents reported suffering from diabetes and hypertension in Chicago compared with Poznań (respectively \( p = 0.006, p = 0.004 \); Table 2) and had a wider knowledge about possibility of simple diagnostic tests purchasing in community pharmacy (\( p < 0.001 \); Table 3). Information about prevention of common chronic diseases were expected by 72.1% of respondents in Poznań and 77.0% in Chicago. Availability of information on common disease prevention was mainly expected by Polish and American respondents in 18-30 and 61-70 year old group (\( p = 0.049, p = 0.002 \), respectively) and by Americans with vocational and graduate education (\( p = 0.011 \); Tab. 4.). Both groups were interested in screening services participating concerning common chronic diseases in community pharmacy.

### RESULTS

The study confirmed that higher percentage of respondents reported suffering from diabetes and hypertension in Chicago compared with Poznań (respectively \( p = 0.006, p = 0.004 \); Table 2) and had a wider knowledge about possibility of simple diagnostic tests purchasing in community pharmacy (\( p < 0.001 \); Table 3). Information about prevention of common chronic diseases were expected by 72.1% of respondents in Poznań and 77.0% in Chicago. Availability of information on common disease prevention was mainly expected by Polish and American respondents in 18-30 and 61-70 year old group (\( p = 0.049, p = 0.002 \), respectively) and by Americans with vocational and graduate education (\( p = 0.011 \); Tab. 4.). Both groups were interested in screening services participating concerning common chronic diseases in community pharmacy.
American respondents indicated it more often for diabetes and hypertension than Poles (p = 0.039, p < 0.001, respectively; Table 5). Poles assumed that such services should be paid by National Health Fund and Americans selected more often community pharmacy as a source of foundation (p < 0.001; Table 6). Participants were also asked to valuate screening service in pharmacy. Respondents in Poznan preferred to pay for these service about 23 złotych while in Chicago 41 USD (157 złotych). In Poznan, the most interested in screenings were participants from age groups 18-30 and 51-60 years old, and with primary education (p < 0.001). In

### Table 4. Effect of age and education on expectation of availability of information about prevention of common chronic diseases in a community pharmacy.

**Question in questionnaire:** Would you like your community pharmacy provide information on prevention of most common public diseases?

<table>
<thead>
<tr>
<th></th>
<th>Yes n (%)</th>
<th>No n (%)</th>
<th>I don't know n (%)</th>
<th>Total n (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POZNAŃ</strong> Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-30</td>
<td>70 (81.4)</td>
<td>8 (9.3)</td>
<td>8 (9.3)</td>
<td>86 (100.0)</td>
<td>0.049</td>
</tr>
<tr>
<td>31-40</td>
<td>24 (70.6)</td>
<td>9 (26.5)</td>
<td>1 (2.9)</td>
<td>34 (100.0)</td>
<td></td>
</tr>
<tr>
<td>41-50</td>
<td>38 (63.3)</td>
<td>9 (15.0)</td>
<td>13 (21.7)</td>
<td>60 (100.0)</td>
<td></td>
</tr>
<tr>
<td>51-60</td>
<td>36 (65.5)</td>
<td>7 (12.7)</td>
<td>12 (21.8)</td>
<td>55 (100.0)</td>
<td></td>
</tr>
<tr>
<td>61-70</td>
<td>16 (84.2)</td>
<td>1 (5.3)</td>
<td>2 (10.5)</td>
<td>19 (100.0)</td>
<td></td>
</tr>
<tr>
<td>&gt; 70</td>
<td>7 (63.6)</td>
<td>2 (18.2)</td>
<td>2 (18.2)</td>
<td>11 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>191 (72.1)</td>
<td>36 (13.6)</td>
<td>38 (14.3)</td>
<td>265 (100.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>4 (100.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>4 (100.0)</td>
<td>0.056</td>
</tr>
<tr>
<td>Vocational</td>
<td>23 (71.9)</td>
<td>2 (6.2)</td>
<td>7 (21.9)</td>
<td>32 (100.0)</td>
<td></td>
</tr>
<tr>
<td>High school</td>
<td>39 (62.9)</td>
<td>10 (16.1)</td>
<td>13 (21.0)</td>
<td>62 (100.0)</td>
<td></td>
</tr>
<tr>
<td>College</td>
<td>57 (85.0)</td>
<td>5 (7.5)</td>
<td>5 (7.5)</td>
<td>67 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Graduate</td>
<td>68 (68.0)</td>
<td>19 (19.0)</td>
<td>13 (13.0)</td>
<td>100 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>191 (72.1)</td>
<td>36 (13.6)</td>
<td>38 (14.3)</td>
<td>265 (100.0)</td>
<td></td>
</tr>
<tr>
<td><strong>CHICAGO</strong> Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-30</td>
<td>38 (88.4)</td>
<td>5 (11.6)</td>
<td>0 (0.0)</td>
<td>43 (100.0)</td>
<td>0.002</td>
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<tr>
<td>31-40</td>
<td>26 (76.5)</td>
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<td>1 (2.9)</td>
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<td>41-50</td>
<td>34 (77.3)</td>
<td>6 (13.6)</td>
<td>4 (9.1)</td>
<td>44 (100.0)</td>
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<tr>
<td>51-60</td>
<td>21 (72.4)</td>
<td>3 (10.3)</td>
<td>5 (17.3)</td>
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<td></td>
</tr>
<tr>
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<td>1 (3.6)</td>
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<tr>
<td>&gt; 70</td>
<td>2 (22.2)</td>
<td>4 (44.5)</td>
<td>3 (33.3)</td>
<td>9 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>144 (77.0)</td>
<td>29 (15.5)</td>
<td>14 (7.5)</td>
<td>187 (100.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
<td>1 (33.3)</td>
<td>3 (100.0)</td>
<td>0.011</td>
</tr>
<tr>
<td>Vocational</td>
<td>16 (94.1)</td>
<td>0 (0.0)</td>
<td>1 (5.9)</td>
<td>17 (100.0)</td>
<td></td>
</tr>
<tr>
<td>High school</td>
<td>42 (75.0)</td>
<td>9 (16.1)</td>
<td>5 (8.9)</td>
<td>56 (100.0)</td>
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</tr>
<tr>
<td>College</td>
<td>23 (59.0)</td>
<td>10 (25.6)</td>
<td>6 (15.4)</td>
<td>39 (100.0)</td>
<td></td>
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<tr>
<td>Graduate</td>
<td>62 (86.1)</td>
<td>9 (12.5)</td>
<td>1 (1.4)</td>
<td>72 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>144 (77.0)</td>
<td>29 (15.5)</td>
<td>14 (7.5)</td>
<td>187 (100.0)</td>
<td></td>
</tr>
</tbody>
</table>

p < 0.05
Table 5. Respondents’ interest in participation in health screening services for diabetes and hypertension in a community pharmacy.

<table>
<thead>
<tr>
<th>Patients’ diseases:</th>
<th>Poznań (n) (%)</th>
<th>Chicago (n) (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>137 (79.2)</td>
<td>89 (89.0)</td>
<td>0.039</td>
</tr>
<tr>
<td>No</td>
<td>36 (20.8)</td>
<td>11 (11.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>173 (100.0)</td>
<td>100 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>118 (68.2)</td>
<td>96 (96.0)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No</td>
<td>55 (31.8)</td>
<td>4 (4.0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>173 (100.0)</td>
<td>100 (100.0)</td>
<td></td>
</tr>
</tbody>
</table>

p < 0.05

Table 6. Preferences for source of funding for health screening services in a community pharmacy.

<table>
<thead>
<tr>
<th>Anwers:</th>
<th>Poznań (n) (%)</th>
<th>Chicago (n) (%)</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>National Health Fund / Government</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>225 (93.0)</td>
<td>84 (45.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No</td>
<td>17 (7.0)</td>
<td>99 (54.1)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>242 (100.0)</td>
<td>183 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Community pharmacy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>10 (4.1)</td>
<td>27 (14.7)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>No</td>
<td>232 (95.9)</td>
<td>156 (85.3)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>242 (100.0)</td>
<td>183 (100.0)</td>
<td></td>
</tr>
</tbody>
</table>

p < 0.05

Chicago, the highest interest was in age group 51-60 years old, with high school education (p < 0.001; Table 7). Additional analysis of results by age, gender and education didn’t achieve the level of statistical significance.

**DISCUSSION AND CONCLUSION**

The need for implementation of screening services was confirmed on the basis of patients’ opinions in this study. American respondents indi-
culated it more often than Poles for diabetes and hypertension. Maybe it is because of the fact that Americans more frequently suggested that they were suffering from diabetes and hypertension. International Diabetes Federation shows in turn that there is 39 million of diabetes in America and 52 million in Europe (6). There is also about 40.0% of European and 36.0% of American population with a higher blood pressure (2). Individuals participating in community pharmacy screening services in Poland were more diverse; it is likely some of them could have undiagnosed diseases. According to global statistic there are about 33.1% of undiagnosed diabetes in Poland and 27.1% in North America (6).

Participants in this study were also interested in education about prophylaxis and prevention of common chronic diseases. Previous studies showed interest to learn about these conditions and that pharmacists were important part of health care system (7, 8). Based on the survey conducted among the pharmacists in 2011 after the screening campaign, the screening process took < 10 min for 3% of participants, 10–15 min for 27%, 15–20 min for 50%

### Table 7. Effect of age and education on preference for cost of health screening services in a community pharmacy.

**Question in questionnaire:** How much in your opinion should be the cost for screening on common diseases performed in a community pharmacy?

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean value (złoty)</th>
<th>SD</th>
<th>Min value (złoty)</th>
<th>Max value (złoty)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POZNAN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-30</td>
<td>51</td>
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<td>15.8</td>
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p < 0.05
32%, 20–25 min for 20%, 25–30 min for 14%, and >30 min. for 4%. Pharmacists stated that nearly all participants were very satisfied with the screening and the quality of information provided (9). In addition, lifestyle modifications through diet alternations and exercise improvement, education can delay or prevent progression from impaired glucose tolerance to type 2 diabetes or hypertension presence (10). Screening and counselling in community pharmacies of individuals at risk for diabetes may result in changes in lifestyle and body weight (11). Moreover, multidisciplinary screening and interventions by pharmacists and also nurses resulted in reduced blood pressure in patients with diabetes (12).

The sequential screening method for diabetes is reasonable in terms of cost and effectiveness (13). Diabetes and hypertension pharmacy-based screening was more costly, but the success rate for referral was higher compared with a community-based service (14). In this study, Poles preferred that such services should be paid by National Health Fund while Americans preferred community pharmacy to pay costs of screening services. Respondents in Poznań determined the sum as 23 złotych and in Chicago as 41 USD (157 złotych). The most interested in paying were Poles in the age groups 18-30 and 51-60 years old, with primary education. In Chicago, participants 51-60 years old with high school education were interested in paying higher cost. Krass and colleagues reported the real total cost of screening each person in a pharmacy was AUS $ 7.76 (about 23 zlotys) for the tick test only and AUS $ 11.83 (about 35 zlotych) for the sequential screening – tick test only and fingertip test for capillary blood glucose service and consisted of variable and fixed costs (13).

Screening programs for chronic conditions have limitations, including substantial cost, limited participation, false-positive cases, false reassurance for negative cases, and potential for social inequity (15, 16). Community pharmacy screening services can help increase health awareness and identify new cases of disease (17-19). The limitation of conducted study is the sample because it is not representative of the general population in Poland and United States of America. The study sample consisted of participants from Poznań and Chicago. Thus, the results may not be generalizable to populations other than the study sample. The respondents’ willingness to pay for screening services was a subjective opinion on the amount they may agree to pay not an actual transaction data.

There is no data from Polish studies about health screenings in community pharmacy so this investigation provides first set of opinions about such a program in Poland. The increased respondents’ willingness to benefit from health screening services should encourage pharmacists to develop and provide screenings in community pharmacies. The results of the study support the need for health screenings in a community pharmacy. Future investigation is needed to understand how to effectively organize and finance these community pharmacy services in Poland.

**Acknowledgment**

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**REFERENCES**


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16 Dluga St.
00-238 Warsaw
Poland

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