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 analysis fills 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...

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 spectroscopy. 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. quinquefolius*, (24). 14 saponins in red *P. ginseng*, (25) and 19 saponins in black ginseng (26).

Charged aerosol detection (CAD)

Nevertheless, ELASD 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 distinction, 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 acclaimed 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 formula. 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 synergic 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 chinensis* 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/HPLC (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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Received: 31. 08. 2016