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APPLICATION OF THIN-LAYER CHROMATOGRAPHY FOR THE QUALITY CONTROL AND SCREENING THE FREE RADICAL SCAVENGING ACTIVITY OF SELECTED PHARMACUETICAL PREPARATIONS CONTAINING *SALVIA OFFICINALIS* L. EXTRACT

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Abstract: Thin-layer chromatographic method, with postchromatographic derivatization, was applied for the purposes of the quality control of pharmaceutical preparations, containing *S. officinalis* L. extract. Six finished products underwent the analysis: capsules, tablets, two ointments, tincture and finished product being a mixture of ethanolic *S. officinalis* and *Thymi vulgaris* extracts. Chromatographic and free radical scavenging fingerprints, obtained for the herbal products, were compared with the profiles of the authenticated botanical reference material. The application of the proposed technique revealed most of the fingerprints, developed for the analyzed preparations, matched with the profiles obtained for authenticated plant material. The developed method was found suitable for the quality control of herbal preparations containing sage extract.

Keywords: *Salvia officinalis* L., thin-layer chromatography, free radical scavenging activity, fingerprints, pharmaceuticals' quality control

Salvia is a large plant genus, comprising ca. 900 species, of the family *Lamiaceae*, which are cultivated worldwide mainly for medicinal and culinary usage (1). The extensive use of sage decoctions and preparations is based on the fact they are rich in biologically active constituents. Plants belonging to the *Salvia* genus are an important source of essential oils used in medicine and perfumery (2). Scientific research indicates that some constituents, present in the *Salvia* essential oil possess antibacterial and antifungal properties. Some compounds demonstrated also anticholinesterase, antioxidant, estrogenic and sedative effects, all of which are currently relevant to the treatment of Alzheimer's disease (3). Several reports indicate that *Salvia* essential oil may also exhibit toxic properties, due to the presence of camphor, α- and β-thujone (4). A strong correlation was found between the contents of these monoterpenes and the oil toxicity (4). *Salvia* genus is a rich source of biologically active water soluble components, namely phenolic acids and flavonoids. Caffeic acid and its depside, rosmarinic acid, are believed to be the major phenolic compounds responsible for the biological activity of *Salvia* samples (1). The polyphenols possess a variety of biological properties, e.g.,: antioxidant, antiplatelet, antitumor and antiviral activity (1). Due to the fact that the reactive oxygen species (ROS) were found responsible for the numerous human diseases (e.g., atherosclerosis, Alzheimer's disease, inflammation, asthma, rheumatoid arthritis, etc.), antioxidants and free radical scavengers are currently the subject of an intensive research interest (5). Our latest research revealed, that extracts obtained from dried sage herbs contain components characterized by a wide polarity range (6). Four species, *S. officinalis*, *S. triloba*, *S. canariensis* and *S. lavandulifolia* were found to possess the greatest amount of antiradicals. The aforementioned study revealed that *S. trilob*a and *S. officinalis* produced almost identical chemical and free radical scavenging fingerprints. It was concluded that it seems quite sensible to introduce *S. triloba* as *S. officinalis* equivalent for the medical purpose, as common sage is the only pharmacopoeial species in Poland. Due to the growing interest in plant derived antioxidants and free radical scavengers, there is a need to screen plant extracts and preparations for their presence. Usually spec-

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trophotometric techniques, with use of a stable free radical DPPH (2,2-diphenyl-1-picrylhyrazyl), are applied to assess total free radical scavenging activity of the investigated sample (7, 8).

Thin-layer chromatography still remains an important tool in the analysis of plant extracts and herbal preparations. Planar chromatography, along with other chromatographic techniques, are commonly applied for constructing fingerprints for the quality control of plant extracts and plant derived products (9–11). For example, chromatographic profiles of twenty sage species, growing and cultivated in Poland, were developed by means of GC-MS (gas chromatography – mass spectrometry) and thin-layer chromatography (11, 12). Recently, the concept of using TLC for investigating biological activity of constituents, present in complex natural samples, has gained much attention (13, 14). It can be attributed to the commonly known advantages of planar chromatography, namely its flexibility, high sample throughput and the speed of method development. It is particularly well suited for the direct biological detection, since the separation result is immobilized prior to the detection and moreover, the open solid bed layer allows direct access to the sample (15).

There are several pharmaceutical preparations, sold over the counter in Poland, containing *Salvia officinalis* extract, including e.g.,: capsules, tablets, tinctures or ointments. Some of them are so called dietary supplements, and as such do not undergo strict quality control. In this paper an attempt was made to develop a thin-layer chromatographic method for the quality control of those preparations. Chemical and free radical scavenging profiles obtained for the finished products were compared with a fingerprint of a botanically identified raw plant material (*S. officinalis*).

EXPERIMENTAL

Apparatus and reagents

All the solvents used for extraction or as the mobile phase components were manufactured by Merck (KGaA, Darmstadt, Germany). Sulfuric acid was obtained from Polish Reagents (Gliwice, Poland). All solvents were of the analytical purity grade. Vanillin and DPPH were purchased from Aldrich.

TLC was performed on the 10 cm × 20 cm glass-backed silica gel TLC $60F_{254}$ plates, purchased from Merck.

Solutions of plant extracts and preparations were applied to the chromatographic plates bandwise by means of a Camag automatic TLC sampler (Camag, Muttenz, Switzerland) and developed in the horizontal DS chambers (Chromdes, Lublin, Poland). Location of the bands was carried out in visible light after derivatization. Chromatograms were documented with use of the Camag TLC Reprostar 3 device with Videostore computer program. The obtained videoscans were processed with use of the Image J image processing program (available on-line, and elaborated by the National Institutes of Health, USA).

Salvia officinalis extract

The extract was obtained from the plants collected in the Pharmacognosy Garden, Lublin, Poland in the course of the vegetation periods of 2007 and 2008. Botanical material was authenticated and voucher specimens were deposited at the Department of Pharmacognosy, Medical University of Lublin, Poland. The dried material was oven-dried at the temperature not exceeding 40°C for 40 h. The plant material remained frozen until the time of extraction and chromatographic analysis. Accelerated solvent extraction (ASE) was performed with the ASE extractor (Dionex, Sunnyvale, CA, USA). Firstly, the samples were extracted with *n*-hexane to get rid of the water-insoluble compounds, then extraction was performed with methanol. The optimized extraction conditions were: temperature, 40°C (*n*-hexane) and 100°C (methanol); pressure, 68 atm (*n*-hexane) and 65 atm (methanol); initial heating time, 10 min (*n*-hexane) and 2 min (methanol); heating time after introduction of solvent, 5 min; static extraction time – 5 min; solvent volume, 40 mL; the number of cycles, 2 (with both solvents). The detailed description of the extraction procedure of the plant material can be found in our earlier work (11).

Botanicals – sample preparation

The following botanicals underwent the analysis: ointments (product I and II), tincture (III), capsules (IV), tablets (V) and finished product being a mixture of *S. officinalis* and *T. vulgaris* extracts. The ointments are used as anti-inflammatory and antiseptic drugs, capsules and tablets can be taken as antiseptics, mainly for curing stomach disorders, while preparations III and VI are applied as antiseptics and anti-inflammatory preparations. In all cases free radical scavenging potential may be responsible, at least partially, for the curing effects.

The finished products I and II were extracted with methanol. The samples were prepared as follows – 10 grams of each ointment was mixed, in a mortar, with three portions of 30 mL methanol. The ointment basis of the product I completely dis-

solved, in the solvent used for extraction, while the basis of preparation II did not and was removed from the extract. The each portion was quantitatively transferred to a calibrated flask, which finally was filled with methanol up to 100 mL.

Tincture (preparation III) and botanical VI, were directly applied to a chromatoplate without any pretreatments.

Capsules (IV) – each capsule contained 450 mg *Salviae* extractum siccum. The contents of four capsules was mixed in a mortar with three portions of 30 ml methanol, and extracted. Three portions were then mixed and filtered through a membrane filter to a calibrated flask, which was finally filled with methanol up to 100 mL.

Tablets (V) – each tablet contained 150 mg *Salviae* extractum siccum. Ten tablets were carefully powdered in a mortar with a pestle. Subsequently, the obtained powder was mixed with three portions of 30 mL methanol, and extracted. Three portions were then mixed and filtered through a membrane filter to a calibrated flask, which was finally filled with methanol up to 100 mL.

The names of the investigated preparations as well as the producers' names are available from authors.

Thin-layer chromatography

The extract of botanically authenticated material and all the samples of the investigated preparations were applied to the 10 cm × 20 cm silica gel TLC plates band-wise (band length 12 mm, 1 μL s^{-1} delivery speed, the track distance 1 mm, distance from the left edge 10 mm and from the low edge 10 mm). Ten-μL aliquots of all the samples were applied onto the plates. The plates were dried in a hood for 10 min before the development. The development was carried out in the horizontal DS chambers at ambient temperature of 20 ± 1°C. For the less polar constituents, mobile phase solvent system I, composed of toluene-ethyl acetate-formic acid, 60:40:1 (*v*/*v*/*v*), was used. For the medium and highly polar substances, solvent system II was applied composed of ethyl acetate-water-formic acid-acetic acid, 100:26:11:11 (*v*/*v*/*v*/*v*). Pre-saturation of the chromatographic chamber was performed only in the case of solvent system II and it lasted for 15 min. The plates were developed to the distance of 90 mm. Then the plates were dried at room temperature for 15 min, prior to derivatization.

Derivatization and DPPH assay

The vanillin-sulfuric acid reagent was prepared by mixing 1 g of vanillin with 20% sulfuric acid in methanol to produce 1% (w/w) solution. The plates were sprayed with this reagent using the automatic spraying device (Merck) and then heated for 5 min at 105°C. Plate images were collected in visible light. They were also scanned with densitometer at the wavelengths λ = 254 and 366 nm, with the slit parameters 1 mm × 0.1 mm.

In order to check free radical scavenging properties of the investigated samples, the chromatographic plates were sprayed with an 0.2% DPPH reagent in methanol and left at ambient temperature for 30 min. Yellow spots obtained as a result of bleaching the purple color of the DPPH reagent were interpreted as a positive free radical scavenging activity (8). Images of the plates were processed with the Image J processing program, and the substances with free radical scavenging activity were identified as negative peaks.

RESULTS AND DISCUSSION

In the first part of the experiment the analyzed samples were chromatographed with use of eluents previously optimized for the resolution of less polar and semi-polar compounds (system I) and for the separation of high polar constituents (system II) (6, 11). The developed plates were sprayed with vanillin-sulfuric acid reagent, the results were documented and used for the comparison of the obtained profiles with the fingerprint of the authenticated reference material. As far as the fingerprints of the polar and semi-polar compounds are considered (Fig. 1a), in almost all the investigated samples the presence of rosmarinic acid was confirmed (R_F = 0.06). The band corresponding to rosmarinic acid was indicated with an arrow. This dimer of caffeic acid is the most abundant phenolic compound in *Salvia* species (1). As it can be noticed in Figure 1a, almost identical profiles were obtained for the tincture (III) and the botanical reference material, sample No. 1 (very similar set of bands was observed for both samples). It confirms the good quality of the finished product and gives certainty that the tincture was prepared from *Salvia officinalis* herb, as labeled by the manufacturer. The chemical profile obtained for preparation I (ointment) (Fig. 1a) was also similar to the *S. officinalis* fingerprint. The only difference is the presence of an intensive dark band (R_F = 0.1), seen in the chromatogram of the finished product. In case of preparation II only less polar constituents were present. As it can be seen in Figure 1a, there are no bands with lower R_F values in preparation's II profile. Chemical fingerprints obtained with solvent system I, for capsules and tablets (prep.

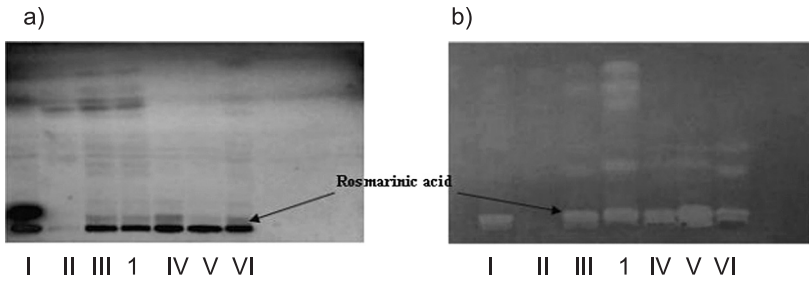

Figure 1. Chemical (a) and free radical scavenging profiles (b) of compounds resolved in the investigated preparations, with use of solvent system I. For symbols, see Experimental

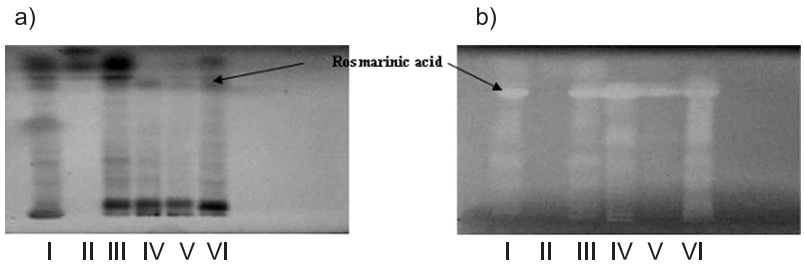

Figure 2. Chemical (a) and free radical scavenging profiles (b) of compounds resolved in the investigated preparations, with use of solvent system II. For symbols, see Experimental

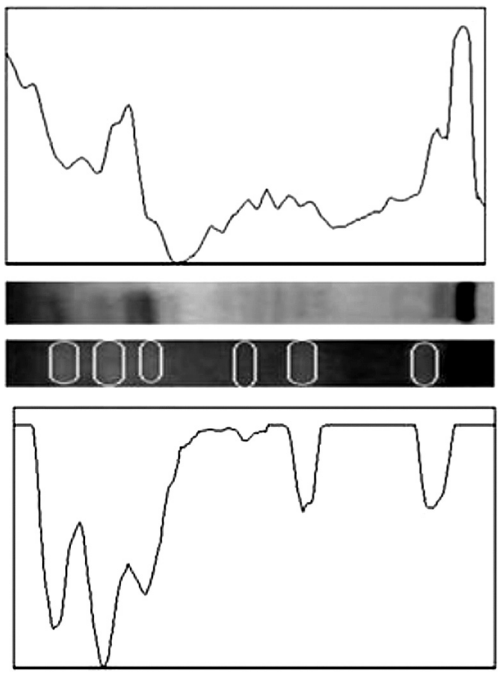

Figure 3. Comparison of chemical and free radical scavenging TLC fingerprints obtained with use of solvent system I, for the *Salvia officinalis* tincture

IV and V) did not contain substances, in the upper R_F range (Fig. 1a). In case of the fingerprints obtained after use of solvent system II, almost all the samples were characterized by very similar profiles (Fig. 2a). However, as it can be easily noticed in Figure 2a, preparation II did not contain any constituents characterized by high polarity values. Lesser amount of high polar compounds was also observed in case of preparation V (tablets). The presence of band, corresponding to rosmarinic acid (R_F = 0.70), was confirmed in five analyzed samples, with an exception of finished product II.

The second part of our study focused on a scrutiny of free radical scavenging properties with the separated constituents, present in the finished products. To this effect, the developed plates were sprayed with the DPPH solution in methanol. Substances that exhibit antiradical potential appeared on the chromatograms as yellow zones on the purple background (Figs. 1b and 2b). Very intensive yellow band corresponding to rosmarinic acid was very characteristic for the majority of the free radical scavenging profiles (as indicated in Figs. 1b and 2b). It was confirmed that this polyphenolic compound is one of the most potent antiradi-

cals present in sage preparations. The antioxidant fingerprints of preparations I and III were similar to those of *S. officinalis* extract, thus the whole complex of free radical scavengers was present in these medicines. In case of tablets and capsules, very potent antioxidants were present in the fingerprints obtained after application of solvent system I (Fig. 1b). However, in both products, as well as in the preparation VI, a lack of very potent free radical scavengers in the upper R_F range (solvent system I) was observed. The use of both, chemical and free radical scavenging fingerprints, gives the possibility to control the samples more comprehensively. As it can be seen in Figure 3, the use of an image processing program, enables the comparison of both profiles, as well as obtaining real chromatograms out of the videoscans. The identity of the resolved compounds does not have to be known, as the similarity degree of the obtained fingerprints can easily be confirmed by use of this program.

CONCLUSIONS

The proposed method can be applied for the quality control of finished products containing sage extract. Different extraction procedures, during the production process of botanicals may lead to the loss of some of the active ingredients. The fingerprints of such products differ from those obtained for the authenticated botanical reference material, as it was observed for sample II. The application of an image processing program eases the comparison of the investigated samples' fingerprints.

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