Original Article

Development of High-performance Thin Layer Chromatography Method for Identification of Phenolic Compounds and Quantification of Rosmarinic Acid Content in Some Species of the Lamiaceae Family

Mariia Shanaida¹, Izabela Jasicka-Misiak², Ewa Makowicz³, Natalia Stanek³, Volodymyr Shanaida³, Piotr P. Wieczorek²

¹Department of Pharmacognosy and Medical Botany, I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine, ²Department of Analytical and Ecological Chemistry, Faculty of Chemistry, University of Opole, Opole, Poland, ³Department of Designing Metal-Cutting Machine and Tools, FabLab Centre, Ternopil Ivan Puluj National Technical University, Ternopil, Ukraine

Aim: Representatives of Nepetoideae Burnett subfamily are promising species of the Lamiaceae Martinov family because of accumulating such valuable groups of secondary metabolites as terpenoids and polyphenols. A high-performance thin layer chromatography (HPTLC) fingerprint method for the qualitative determination of phenolic compounds and for the quantification of rosmarinic acid (RA) content in methanol extracts of five species of this subfamily was developed for the first time. Materials and Methods: Dried aerial parts of Dracocephalum moldavica L., Ocimum americanum L., Lophanthus anisatus (Nutt.) Benth., Monarda fistulosa L., and Satureja hortensis L. collected in flowering period were macerated with methanol. The HPTLC analysis was conducted using the CAMAG analytical system (Muttenz, Switzerland). The comparative analysis of RA contents was performed by HPTLC densitometric detection at $\lambda = 366$ nm. Results: Identification of polyphenols in the investigated herbs was performed by comparison of a color and $R_f$ of the chromatographic zones with six reference standards: rutin, apigenin, luteolin, caffeic acid, chlorogenic acid, and RA. HPTLC method was also validated for the quantification of RA in the extracts of investigated herbs. RA contents decreased in such a sequence: $D. moldavica$ (24.83 ± 0.78 mg/g) > $M. fistulosa$ (20.32 ± 0.64 mg/g) > $O. americanum$ (19.59 ± 0.61 mg/g) > $S. hortensis$ (18.77 ± 0.52 mg/g) > $L. anisatus$ (12.61 ± 0.43 mg/g). Conclusion: Obtained data can facilitate the differentiation of investigated species using the chromatographic fingerprints of their phenolic compounds. Developed and validated HPTLC method provides an approach to estimate RA content as a common marker of investigated herbs.

Keywords: chromatographic fingerprints, high-performance thin layer chromatography, Lamiaceae, phenolic compound, rosmarinic acid

INTRODUCTION

A large number of medicinal plants are nowadays used all over the world for healing purpose and for the development of new drugs.[1-3] Phytochemical analysis is necessary for understanding the therapeutic role of natural extracts or their compounds and in the case of standardization of crude drugs.[3]

Address for correspondence: Dr. Mariia Shanaida, Department of Pharmacognosy and Medical Botany, I. Horbachevsky Ternopil National Medical University, Volii Street 1, 46001, Ternopil, Ukraine. E-mail: shanayda@tdmu.edu.ua

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medicinal properties; they are rich in terpenoids and polyphenols with valuable healing function.\textsuperscript{4-9} There are many famous genera of medicinal plants (\textit{Mentha, Melissa, Origanum, Rosmarinus, Salvia, Thymus, etc.}) belonging to the Nepetoideae subfamily. Polyphenols play a key role in the defense mechanism of plant body against the harmful influence of ultraviolet (UV) radiation, predators, microorganisms, and so on.\textsuperscript{10,11} Rosmarinic acid (RA) is the most widespread among polyphenols of Nepetoideae subfamily;\textsuperscript{12} so, there is a need of simple and rapid analytical method for its evaluation in the representatives of the subfamily.\textsuperscript{5,13}

Unlike synthetic drugs, plants are very complex mixtures of chemical compounds. The chemical composition of plants depends to a large extent on the growing condition, chemotype, and origin of the plant raw material; the choice of the extraction procedures is also very important for evaluating different compounds.\textsuperscript{14,15} In the case of plants not included in Pharmacopoeias, such as representatives of genus \textit{Dracocephalum, Monarda, Lophanthus, Ocimum, and Satureja}, the developing of specification for the new herbal plant materials by chromatographic methods plays a very important role.

Different analytical techniques are used for detecting phenolic compounds in medicinal plant materials, such as thin layer chromatography (TLC), and high-performance thin layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), and liquid chromatography–mass spectrometry. Flavonoids and phenolic acids are often analyzed by use of the similar chromatographic systems because of their common physicochemical properties allowing them to be extracted together.\textsuperscript{16}

A chromatographic fingerprint of plant extracts using the HPTLC represents a chromatographic pattern of pharmacologically active or chemically specific constituents available in the plants.\textsuperscript{14} HPTLC is an efficient and automated form of TLC having the latest technical developments for quality assessment and evaluation of botanical materials, including representatives of the Lamiaceae family\textsuperscript{4,6,13,16,17} because of the advantages such as ease of sample preparation, optimization of fingerprint for certain compounds, and comparison of many samples on a single plate. Comparison of the whole chromatograms gives a possibility to reveal the trace similarities and dissimilarities among the investigated plants.\textsuperscript{18}

Variety of extraction methods, solvents, mobile phases, and derivatization procedures are used for the identifications of phenolic compounds in plants belonging to the Nepetoideae subfamily of Lamiaceae family (genera \textit{Melissa, Mentha, Thymus, Salvia, Origanum, Draccocephalum, Monarda, Ocimum, etc.}) by TLC and HPTLC.\textsuperscript{14,16,13,17,18} However, there are few reported data about applying of this method for species of this family which are potentially perspective for medicinal purpose, especially in the case they are not at present included in Pharmacopoeias.\textsuperscript{1,2} HPTLC method was not always validated by researchers to analyze the content of polyphenol components in Lamiaceae family.\textsuperscript{19} Recently, the antioxidant effects of polyphenols obtained from Lamiaceae representatives have also been studied.\textsuperscript{4,5,9} Natural antioxidants can be considered as an alternative to the synthetic ones, which can be carcinogenic and toxic in high doses.\textsuperscript{19}

The purpose of this study was to develop the HPTLC method for the identification of phenolic compound and the quantification of RA in methanol extracts of \textit{Dracocephalum moldavica, Ocimum americanum, Lophanthus antisatus, Monarda fistulosa, and Satureja hortensis} herbs.

**Materials and Methods**

**Plant material**

The aerial parts of \textit{D. moldavica, O. americanum, L. antisatus, M. fistulosa, and S. hortensis} were collected at the flowering stage from the experimental plots in Ternopil region (Ukraine) in 2018. Herbs were dried in shadow at 25°C–35°C and placed in tightly closed containers till further use. Voucher specimens of plants are kept at the Department of Pharmacognosy and Medical Botany of I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine.

**Chemicals and materials**

All chemicals and reagents used were of analytical grade. Chloroform, ethyl acetate, methanol, formic acid, and AlCl\textsubscript{3} were purchased from POCH S.A. (Gliwice, Poland). Commercially available standards of rutin, apigenin, luteolin, caffeic acid, chlorogenic acid, and RA were purchased from Sigma-Aldrich (Poznan, Poland). HPTLC analyses were performed on 20 cm × 10 cm HPTLC silica gel 60 F\textsubscript{254} plates (Merck, Darmstadt, Germany).

**General experimental procedures of high-performance thin layer chromatography**

Extraction of phenolic compounds was performed as was described by Altan \textit{et al.}\textsuperscript{13} with some modifications. The powdered plant material (1.0 g) was macerated for over 24 h in methanol (5 mL) at room temperature (22°C ± 1.0°C); it was also shaken for 2 h in Vibramax 100 at 450 rotation per minute (rpm). Methanol extracts
were filtered through the paper filter and placed in the volumetric flasks (50 mL), then methanol was added to the mark. Obtained extract (2 mL) was filtered through a 0.45 μm Millipore filter and was used for analysis as test solution. A stock standard solution (250 μg/mL) of each phenolic compound was prepared in methanol by weighing out 1 mg of the analyte into 4 mL of methanol; 5 μL of each stock standard solution was used for the HPTLC analysis.

Chromatographic analyses were carried out on a CAMAG analytical system (Muttenz, Switzerland). The HPTLC method was performed as described by Stanek et al. Extracts (12 μL) and standard solutions (5 μL) were applied to HPTLC plates using an automatic HPTLC application device (Linomat 5, CAMAG). Appropriate concentrations of the chromatographed compounds were determined from the intensity of diffusely reflected light.

Chromatographic separation was performed on 20 cm × 10 cm HPTLC plates through vertical glass chamber (CAMAG). Mobile phases were as follows: (1) chloroform:ethyl acetate:formic acid (5:4:1) and (2) ethyl acetate:formic acid:water (15:1:1). A total of 85 mL of mobile phases were used per chromatography, and each time they were prepared freshly. The chamber saturation time for mobile phase was at a minimum of 40 min. The length of chromatogram run was 8 cm. Plates were dried in the oven after the development. Detection was based on natural fluorescence before and after the post-chromatographic derivatization by spraying with 1% methanol AlCl3 under UV light at 254 and 366 nm. The densitometric detection for comparative analysis of RA contents was performed by HPTLC at λ = 366 nm using scanning with CAMAG TLC Scanner. Concentration of the RA was determined from the intensity of reflected light.

The chromatographic images [Figures 1 and 2] were obtained at λ = 366 nm using the TLC scanner of CAMAG analytical system. Data acquisition and calibrations were performed with HPTLC software (visionCATS, CAMAG).

Method of validation

Quantitative determination of RA was carried out using the mobile system B at 366 nm in the absorbance mode without derivatization. The proposed method was validated on the basis of the linearity, limit of detection (LOD), limit of quantification (LOQ), and precision.[21]

The “linearity” was evaluated by analyzing RA standard solutions with concentrations from 6.25 to 200 μg/mL using six different calibration doses [Figure 3]. The calibration curve of the RA was made by serial dilution of the stock standard (six sets of standard dilutions). Of which, 5 μL of the obtained stock standard solutions were applied to the same plate to prepare the linear calibration curve. A peak area versus concentration was subjected to least square linear regression analysis, and the slope and correlation coefficient for the calibration was determined. The analysis was performed in triplicate.

Figure 1: High-performance thin layer chromatography fingerprints of herbs and polyphenol standards in mobile phase A: 1, apigenin; 2, luteolin; 3, rutin; 4, caffeic acid; 5, rosmarinic acid; 6, chlorogenic acid; A, Dracocephalum moldavica; B, Lophanthus anisatus; C, Monarda fistulosa; D, Ocimum americanum; E, Satureja hortensis
“LOD” and “LOQ” were determined from the calibration curve:

\[
LOD = 3.3 \cdot SD / a \\
LOQ = 10 \cdot SD / a
\]

where SD is the standard deviation and \(a\) is the slope of the calibration curve.

The “intraday precision” was estimated [Figure 4] by performing replicate analyses of RA samples (100 \(\mu\)g/mL, \(n = 6\)) on the same day. The “interday precision” of the assay was determined by repeating the intraday assay on three different days. The precision was expressed as the relative standard deviation (RSD, %).

Statistical analysis was performed using the software Statistica, version 13.1 (StatSoft, California, USA).

**RESULTS**

HPTLC method was used for evaluating the “chromatographic fingerprints” of individual phenolic
compounds and quantitative determination of RA in the methanol extracts of five Lamiaceae representatives. The maceration process was carried out at room temperature to prevent the degradation of polyphenols in case of the heating influence. The chromatographic conditions (mobile phases, variety of available reference standards, chamber saturation time, run length, sample application volume, etc.) were optimized to give reproducible $R_f$ values. Identification of phenolic compounds was performed by comparison of the color and $R_f$ of the bands with six polyphenol standards (rutin, apigenin, luteolin, caffeic acid, chlorogenic acid, and RA). Aforementioned polyphenol standards were chosen as common phenolic compounds presented in Nepetoideae representatives.

**Qualitative determination of phenolic compounds**

Sequence of the zones present in the chromatograms scanned at 366 nm is shown in Figures 1 and 2, and the result was found to be satisfactory. The chromatogram of all test solutions showed the most intense light blue zones at $R_f = 0.23$ and $R_f = 0.75$ in the mobile systems A [Figure 1] and B [Figure 2], respectively, corresponding to reference RA. Weaker light blue zones of caffeic acid were presented in chromatograms of all the investigated species above the RA spots ($R_f = 0.41$ in mobile phase A and $R_f = 0.79$ in mobile phase B). Weak blue zones corresponding to chlorogenic acid were seen only in the chromatograms of *L. anisatus* and *M. fistulosa* in the mobile system B ($R_f = 0.28$). Furthermore, additional zones in different shades of blue colors were presented specific for each plant positions.

Flavonoids apigenin and luteolin were not identified clearly in the methanol extracts of all investigated plants comparatively with reference standards using both mobile phases. It may confirm the opinion of researchers that aerial parts of plants in flowering period are characterized by the domination of phenolic glycosides over aglycones. Flavonoid rutin was identified clearly only in *O. americanum* using mobile phase B ($R_f = 0.09$).

The main differences between the investigated species were the presence of two yellow doubled zones detected in the middle of *L. anisatus* track in mobile phase B; two other clearly seen yellow zones were evaluated just below and above the position of RA. In the *M. fistulosa* chromatogram, a clearly separated navy blue zone was identified at $R_f = 0.08$ and yellow zone located slightly above the position of caffeic acid at $R_f = 0.42$ in mobile phase A. Analysis of the *M. fistulosa* chromatogram developed in mobile phase B showed three yellow bands in the lower third of the chromatogram, navy blue zone at $R_f = 0.69$, and several light blue ones in the range of $R_f = 0.15–0.38$.

*D. moldavica*, *M. fistulosa*, and *S. hortensis* tracks developed in the mobile phase B were characterized by the clearly seen identical yellow bands at $R_f = 0.25$, whereas in the case of *L. anisatus* and *O. americanum*, these bands were not observed. According to Vronška, such feature on the color and $R_f$ value can be referred to the glycoside of luteolin. On the *S. hortensis* chromatogram in the mobile phase B, the following bands were detected: dark blue one at $R_f = 0.68$ directly under RA zone, and a few weak ones in the lower half of the track. *O. americanum* track in the aforementioned mobile phase is characterized by several well-separated weak blue and yellow bands in the lower part. The least quantity of fluorescent bands was evaluated in *D. moldavica* chromatogram in both mobile phases.

In the upper parts of all test solution tracks, several red zones were seen, especially in the mobile phase A. According to such fluorescent red zones are characteristic of chlorophyll molecules. As can be observed from the obtained results, mobile phase B is more appropriated for the separating of phenolic compounds from the herbs of investigated species.

**Quantitative determination of rosmarinic acid content**

The quantitative determination of RA content established a good correlation coefficient for peak areas of the resolved spots plotted against concentration in the range of 12.5–200 μg/mL. A summary of the validation parameters is presented in Table 1.

The experimental results showed that RA content in the five investigated herbs by HPTLC quantification was found in the ranges of 12.61–24.83 mg/g and decreased in the following sequence: *D. moldavica* (24.83 ± 0.78 mg/g) > *M. fistulosa* (20.32 ± 0.64 mg/g) > *O. americanum* (19.59 ± 0.61 mg/g) > *S. hortensis* (18.77 ± 0.52 mg/g) > *L. anisatus* (12.61 ± 0.43 mg/g).

As investigated plants are not included to Pharmacopoeias, conducted analyses may play an important role in developing the specification to the new representatives including the phytochemical analysis by chromatographic methods.

**Discussion**

The results of this research suggested that several hydroxycinnamic acids and flavonoids are present in the methanolic extracts of *D. moldavica*, *M. fistulosa*, *O. americanum*, *S. hortensis*, and *L. anisatus* herbs. Satisfactory separation of phenolic compounds was
et al. and Janicsák [24] confirmed the findings of this study. Plants [5,9,13] according to the presence of four hydroxyl groups in the molecule. Dominating of RA can be closely related to the high potential of free radicals scavenging activity of these compounds. O. vulgare showed the highest antioxidant capacity comparatively with the other species of Nepetoideae subfamily [24]. Prominent common compound of the investigated herbs similarly to the other species of Nepetoideae subfamily, and it correlated with the most abundant RA and polyphenolic contents in its extract. [5] Antiviral, antibacterial, anti-inflammatory, analgesic, and anxiolytic activities of RA were also proven [12,25,26].

High content of RA (27.2 mg/g) was also present in 70% methanol extracts of cell suspension of D. moldavica herb, and this species was estimated as a good candidate for in vitro production of this compound [27]. The highest level of RA content was also established for the herbal infusion of D. moldavica among the other species of Nepetoideae subfamily by HPLC [8]. High content of this compound was also revealed in the aerial parts of some Salvia species [13].

This study also indicated that caffeic acid was the other common compound of the investigated herbs similarly to the other species of Nepetoideae subfamily [24]. Prominent anti-inflammatory, antioxidative, and antioxidant effects were established for this phenylpropanoid experimentally [22,25]. The results of Fecka and Turek [6] and Janicsák et al. [24] confirmed the findings of this study that caffeic acid often occurs in aerial parts of plants in Nepetoideae subfamily together with its ester RA.

Flavonoid rutin was clearly identified only in O. americanum herb. The presence of four phenolic groups in rutin molecule is known for scavenging the radiation-induced radicals; glycosylation may greatly reduce the vascular relaxation effect in coronary artery; and 4-oxo group and the 2,3 double bond in the C ring may be related by its neuroprotective action [28]. The aerial part of O. basilicum was also characterized by high content of this flavonol glycoside. Therefore, it can be concluded that despite the fact that most of components of flavonoids appearance were not identified in the herbal material of investigated plants in the presence of available reference compounds it opens the perspective for their further isolation and investigation.

It had been reported that high content of phenolic compounds as well as aromatic components in essential oils is well correlated with the antioxidant activity of plant materials of Lamiaceae representatives [4,5,9,27,29,30]. Therefore, according to the foregoing, investigated herbs have a potent antioxidant property due to high content of RA, and the presence of other spots of polyphenols in HPTLC chromatograms.

**Conclusion**

This study revealed the precise identification of phenolic compounds by the HPTLC fingerprint method in methanol extracts of five Nepetoideae representatives: D. moldavica, M. fistulosa, O. americanum, S. hortensis, and L. anisatus. The characteristic chromatographic polyphenol patterns of investigated species were evaluated. Developed HPTLC method can be proposed as a simple and rapid quality control routine analysis of investigated herbs and may provide a reliable tool for the identification of the new plant raw materials or to manage their adulterations. This chromatographic method was also first developed and validated for the quantification of RA content in methanol extracts of investigated herbs. Future research in the area of isolation of bioactive compounds from these herbs responsible for antioxidant activity can give a new direction in their pharmacological research.

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**Conflicts of interest**

There are no conflicts of interest.

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| Table 1: Validation parameters of rosmarinic acid in high-performance thin layer chromatography quantification |
| --- |
| Parameter | Result |
| Regression equation | \( y = 5.687 \times 10^{-3} x + 8.487 \times 10^{-4} \) |
| Correlation coefficient (linearity) | 0.9925 |
| LOD (µg/mL) | 9.63 |
| LOQ (µg/mL) | 29.18 |
| Intraday precision (RSD, %) | 1.13 |
| Interday precision (RSD, %) | 1.47 |

obtained in both mobile systems: chloroform:ethyl acetate:formic acid (5:4:1) and ethyl acetate:formic acid:water (15:1:1).
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