Original article

SIMULTANEOUS DETERMINATION OF PHENOLIC COMPOUNDS IN *MENTHA SPICATA* L. SUBSP. *SPICATA* BY RP-HPLC

Alper GÖKBULUT*, Engin ŞARER

Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, 06100
Tandoğan-Ankara, TURKEY

Abstract

A high performance liquid chromatographic method was applied to the determination of some phenolic compounds in the leaves of Mentha spicata L. subsp. spicata. Phenolic compounds were known as antioxidants so that the profile of such compounds in the plants are taking more attention. In this study the qualitative and quantitative analysis of the methanolic extract of Mentha spicata subsp. spicata growing in Turkey in terms of phenolic compound characterization were performed by RP-HPLC for the first time. The plant contains caffeic acid, rosmarinic acid and luteolin among the investigated seven compounds and especially the rosmarinic acid content of the plant was found so high as 1.34g/100g. The plant was found to be a really good source of rosmarinic acid and it's feasible that standardization of the extract of Mentha spicata subsp. spicata could be done via this developed method over rosmarinic acid due to the significant amount in the plant.

Key words: *Mentha spicata subsp. spicata, phenolic compounds, RP-HPLC*

Mentha spicata L. subsp. *spicata* Bitkisinde Fenolik Bileşiklerin Ters Faz YPSK ile Eş Zamanlı Olarak Tayini

Mentha spicata L. subsp. spicata bitkisinin yapraklarında bulunan bazı fenolik bileşiklerin tayini için yüksek performanslı sıvı kromatografisi yöntemi uygulanmıştır. Fenolik bileşikler antioksidan özellikte olduğundan bitkilerdeki profilleri önem taşımaktadır. Bu çalışmada Türkiye'de yetişen Mentha spicata subsp. spicata bitkisinin metanollü ekstresinin bazı fenolik bileşikler açısından kalitatif ve kantitatif analizleri ters faz YPSK ile ilk kez yapılmıştır. Bitkide; incelenen yedi bileşikten kafeik asit, rozmarinik asit ve luteolol varlığı belirlenmiş olup özellikle 1.34g/100g oranıyla rozmarinik asit miktarının çok yüksek olduğu saptanmıştır. Bitkinin çok iyi bir rozmarinik asit kaynağı olduğu tespit edilmiş olup, Mentha spicata subsp. spicata ekstrelerinin standardizasyonunun, bitkideki yüksek içeriği nedeniyle rozmarinik asit üzerinden, tarafımızca geliştirilen bu analiz yöntemiyle yapılabileceği düşünülmektedir.

Anahtar kelimeler: *Mentha spicata subsp. spicata, fenolik bileşikler, Ters faz YPSK*

*Correspondence: Tel: +903122133106; Fax: +903122131081
E-mail: gokbulut@pharmacy.ankara.edu.tr

INTRODUCTION

The genus *Mentha* (Lamiaceae) is represented by approximately 30 species growing in temperate regions of Eurasia, Australia and South Africa (1,2). The genus is under cultivation from tropical to temperate climate of America, Europe, China, Brasil and India and has really economic importance in the world due to the mint oil. *Mentha spicata* L. (spearmint) is a creeping rhizomatous, glabrous and perennial herb with a strong aromatic odor. The species has been found useful as digestive and gastro-stimulant (3). The leaves of the plant is used as tea flavouring agent. The fresh and dried plants and their essential oils are widely used in food, cosmetic, confectionary, chewing gum, toothpaste and pharmaceutical industries (4,5).

Phenolic compounds, especially hydoxycinnamic acid derivatives are rather widespread in plants and they have attracted a great interest because of their various biological and pharmacological activities including antioxidative, antiviral, antiallergic and antilisterial activities (6-9). The studies were mostly focused on the essential oil composition of *Mentha* species and there are a few studies on the phenolic composition of *M. spicata* (2,10,11). *M. spicata* has two subspecies growing in Turkey and there was no report on the phenolic profile of *M. spicata* subsp. *spicata*. In this study, we aimed to determine some phenolics in the leaves of *M. spicata* subsp. *spicata* qualitatively and quantitatively.

EXPERIMENTAL

Materials

Mentha spicata L. subsp. *spicata* was collected from Malatya at an altitude of 964 m on its flowering time. The leaves of the plant were dried at room temperature away from sunlight. Voucher specimen of the plant has been deposited at the Herbarium of Inonu University, Faculty of Pharmacy (0001).

Chemicals and standards

Chromatographic grade double distilled water, HPLC grade methanol, acetonitrile and analytical grade trifluoro asetic acid were used for the HPLC analysis. All the phenolic compounds were purchased from Sigma. Chlorogenic acid (C3878), caffeic acid (C0625), rosmarinic acid (536954), myricetin (M6760), quercetin (Q4951), luteolin (L9283), kaempferol (K0133).

Extraction

200 mg of dried and powdered leaves of *M. spicata* subsp. *spicata* were extracted with methanol by the aid of magnetic stirrer, for 6 h (50 °C, 250 rpm). The extract was then filtered and completed to 10.0 ml in a volumetric flask with methanol and passed through 0.45 μm filter and injected into the HPLC system.

Apparatus

An Agilent 1100 Series HPLC system with a quaternery solvent delivery system, an online degasser, an autosampler, a DAD dedector was used for the analysis. The column was Phenomenex Luna C_{18} (5 μm, 250 mm X 4.6 mm) and column temperature was maintained at 30 °C. The system was controlled and data analysis were performed by Agilent Chemstation Software. All the calculations concerning the quantitative analysis were performed with external standardization by the measurement of peak areas.

Stock and standard solutions

Chlorogenic acid (10.30 mg), caffeic acid (10.30 mg), rosmarinic acid (5.00 mg), myricetin (1.00 mg), quercetin (10.00 mg), luteolin (1.00 mg) and kaempherol (5 mg) were accurately weighed into a 10 ml volumetric flask, dissolved in methanol and filled up to volume for preparing stock solutions. Standard solutions were prepared in methanol for each phenolic compound at five different concentration levels in 10 mL volumetric flasks for the establishment of calibration curves (Table 1).

Table 1. Linear relationships between peak area and concentration.

Analyte	R T(min)	Standard curve	r^2	LOD (µg/mL)	LOQ (µg/mL)
Caffeic acid	8,1	y=50251x-10.690	0.9989	0.0675	0,225
Rosmarinic acid	12,4	y=22688x-56.030	0.9991	0.0990	0.330
Luteolin	16,9	y=40356x-13.308	0.9999	0.0531	0.177

y: peak area; x: concentration of analyte (µg/mL), r^2: the correlation coefficient of the regression equation, LOD: Limit of dedection S/N:3, LOQ: Limit of quantification S/N:10

Procedure
Chromatographic conditions

The analysis were performed by gradient elution with a flow rate of 1 mL/min. Column temperature was set to 30 °C. The mobile phase was a mixture of trifluoroasetic acid 0.1 % in water (solution A), trifluoroasetic acid 0.1 % in methanol (solution B), trifluoroasetic acid 0.1 % in acetonitrile (solution C). The composition of the gradient was (A:B:C), 80:10:10 at 0 min, 60:25:15 at 5 min, 50:30:20 at 10 min, 40:40:20 at 15 min and 0:75:25 at 20 min (Table 2). The duration between runs was 5 min. All solvents were filtered through a 0.45 µm Milipore filter before use and degassed in an ultrasonic bath.

Table 2. Gradient system for the HPLC analysis.

Minutes	A %	B %	C %	Flow Rate
0	80	10	10	1.0 ml/min
5	60	25	15	1.0 ml/min
10	50	30	20	1.0 ml/min
15	40	40	20	1.0 ml/min
20	0	75	25	1.0 ml/min

Calibration

Standard solutions containing caffeic acid, rosmarinic acid and luteolin were prepared in methanol. Triplicate 5 µl injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area of each drug was plotted against the concentration to obtain the calibration graph.

Limits of detection and quantification

Limits of detection (LOD) were established at a signal to noise ratio (S/N) of 3. Limits of quantification (LOQ) were established at a signal to noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by nine injections of caffeic acid, rosmarinic acid and luteolin at the LOD and LOQ concentrations. The LOD was calculated to be 0.0675, 0.099 and 0.0531 μg/mL and the LOQ was calculated to be 0.225, 0.330 and 0.177 μg/mL for caffeic acid, rosmarinic acid and luteolin, respectively (Table 1).

Precision

The precision of the method (within–day variations of replicate determinations) was checked by injecting nine times of caffeic acid, rosmarinic acid and luteolin at the LOQ levels. The precision of the method, expressed as the RSD % at the LOQ levels were 4.375 %, 3.600 % and 3.082 % for caffeic acid, rosmarinic acid and luteolin, respectively (Table 3).

Table 3. Precision of the method at the LOQ level (n=9).

Compound	λ (nm)	Peak Area (Mean)	RSD %
Caffeic acid	330	9.300	4.375
Rosmarinic acid	330	6.442	3.600
Luteolin	340	8.527	3.082

RSD % = (SD / Mean) X 100, SD = Standart Deviation

RP-HPLC analysis

Volumes of 5 μL of each prepared solutions of samples were injected into the column and the chromatograms were recorded from 200 to 400 nm. Standard solutions were analyzed and three-dimensional chromatograms (wavelength; time; absorbance) were obtained to select the optimum wavelength for detection of these phenolic acids with maximum sensitivity. Quantification was performed by setting the detection wavelength as 330 nm for caffeic and rosmarinic acids and 340 nm for luteolin using photo-diode array detector. The results were obtained as a mean value of three separate injections by using external standard method. The standard solutions of caffeic acid, rosmarinic acid and luteolin were added respectively to extracts and injected. The areas of peaks corresponding to standards were increased to prove the presence of these compounds. The peaks in the chromatograms were identified by comparing the retention times and UV-spectra with three standards.

RESULTS AND DISCUSSION

In the present study, the qualitative and quantitative analysis of phenolic compounds in *M. spicata* subsp. *spicata* growing in Turkey were performed for the first time. The results revealed that chlorogenic acid, myricetin, quercetin and kaempferol were not determined in the plant. Rosmarinic acid, the tanning compound of Lamiaceae, was seemed to be the major phenolic compound in *M. spicata* subsp. *spicata* as 1.344g/100g. Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid and was isolated from many species of the families of Lamiaceae and Boraginaceae as a polyphenol natural antioxidant compound (12).

In one of the limited studies on phenolics of *M. spicata*, Wang et al. found rosmarinic acid as 14.3 mg/g and caffeic acid as 0.3 mg/g in ethanolic extracts of the leaves (11). When we compare with the results obtained from our study on *M. spicata* subsp. *spicata* leaves we can see that rosmarinic acid contents were similar but caffeic acid content was lower in our plant sample. In another study, Dorman et al. investigated aqueous extract of the aerial parts of *M. spicata* var. *crispa* and rosmarinic acid content was found as 4.60 mg/g, while caffeic acid and luteolin contents were found as 0.19 mg/g and 0.54 mg/g, respectively (2). In Wang et. al study, extraction solvents were compared and they indicated that water extracts contained less rosmarinic acid and caffeic acid among the other less polar solvent extracts.

In our study, several proportions of mobile phases including methanol, acetonitrile and water in combination with trifluoroacetic acid were tested. The gradient system given in Table 2 provided the best separation of investigated phenolics. It's clear that a good separation was achieved within 20 minutes using the conditions given above. The chromatograms of the standard mixture of investigated phenolics and of *M. spicata* subsp. *spicata* extract are given in Figure 1 and 2. Contents of caffeic acid, rosmarinic acid and luteolin are given in Table 4.
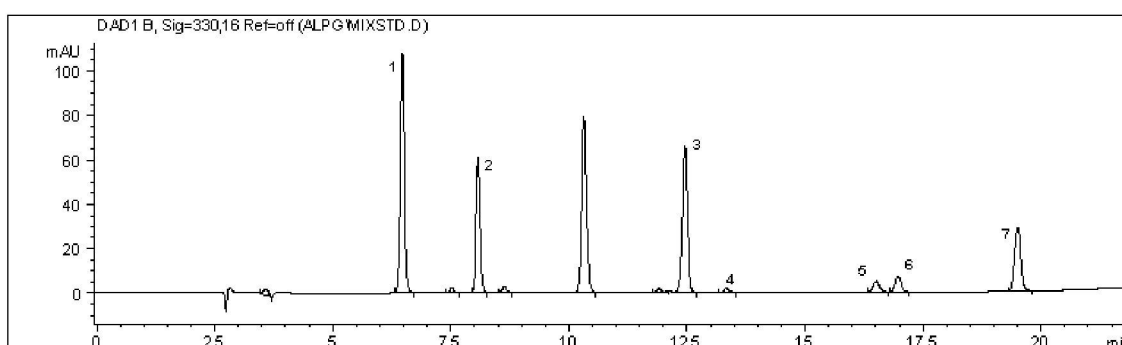

Figure 1. Chromatogram of the standard mixture (1. Chlorogenic acid, 2. Caffeic acid, 3. Rosmarinic acid, 4. Myricetin, 5. Quercetin, 6. Luteolin, 7. Kaempherol).
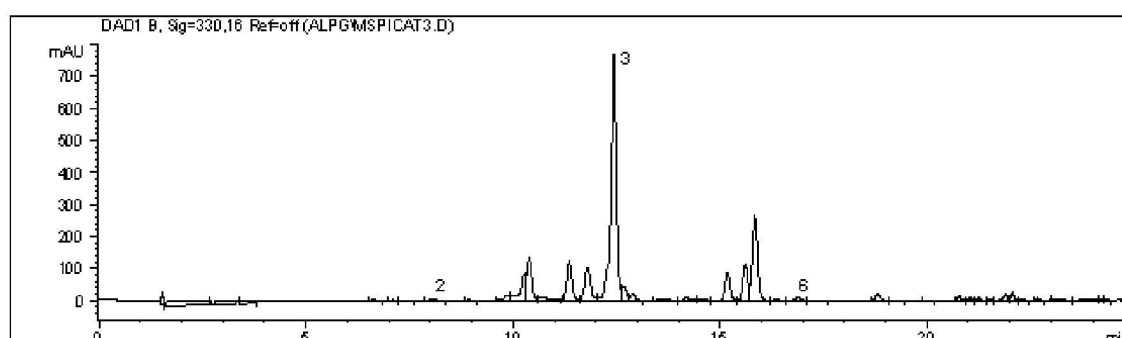

Figure 2. Chromatogram of *Mentha spicata* L. subsp. *Spicata.*

In conclusion, it is clear that *M. spicata* subsp. *spicata* from Turkey has rosmarinic acid in significant amount and a feasible standardization of the extracts of *M. spicata* subsp. *spicata* could be done via this method over rosmarinic acid with this developed accurate, simple and sensitive method.

Table 4. Contents of caffeic acid, rosmarinic acid and luteolin in *Mentha spicata* subsp. *Spicata.*

Sample	Caffeic acid (g/100gdw) n=3, Mean ± SD	Rosmarinic acid (g/100gdw) n=3, Mean ± SD	Luteolin (g/100gdw) n=3, Mean ± SD
Mentha spicata subsp. *spicata*	0.0089± 0.0008 (8.988)*	1.344 ± 0.0085 (0.632)*	0.0168 ± 0.0005 (2.976)*

*RSD % values are given in the parenthesis, RSD % = (Standart Deviation / Mean) X 100, SD = Standart Deviation, dw = dry weight

REFERENCES

1. **Davis, P.H.,** Flora of Turkey and the East Aegean Islands, Vol. 7, pp. 384, Edinburgh University Press, Edinburgh, **1982.**

2. **Dorman, H.J., Kosar, M., Kahlos, K., Holm, Y., Hiltunen, R.,** "Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties and cultivars" *J. Agric. Food Chem.*, 51, 4563–4569, **2003.**

3. **Chauhan, R.S., Kaul, M.K., Shahi, A.K., Kumar, A., Ram, G., Tawa, A.,** "Chemical composition of essential oils in *Mentha spicata* L. accession [IIIM(J)26] from North-West Himalayan region, India" *Ind. Crop. Prod.*, 29, 654-656, **2009.**

4. **Yonis, Y.M., Beshir, S.M.,** "Carvone rich essential oil from *Mentha longifolia* (L) Huds. ssp. Schimperi Briq. and *Mentha spicata* L. grown in Sudan" *J. Essent. Oil Res.*, 16, 539–541, **2004.**

5. **Lawrence, B.M.,** Mint: The Genus *Mentha*, CRC Press, Boca Raton, FL, **2006.**

6. **Kono, Y., Kobayashi, K., Tagawa, S., Adachi, K., Ueda, A., Sawa, Y., Shibata, H.,** "Antioxidant activity of polyphenolics in diets, rate constants of reactions of chlorogenic acid and caffeic acid with reactive species of oxygen and nitrogen" *Biochim. Biophys. Acta*, 1335, 335-342, **1997.**

7. **Chiang, L.C., Chiang, W., Chang, M.Y., Ng, L.T., Lin, C.C.,** "Antiviral activity of *Plantago major* extracts and related compounds *in vitro*" *Antivir. Res.*, 55(1), 53-62, **2002.**

8. **Wen, A., Delaquis, P., Stanich, K., Toivonen, P.,** "Antilisterial activity of selected phenolic acids" *Food Microbiol.*, 20(3), 305-311, **2003.**

9. **Seelinger, G., Merfort, I., Schempp, C.M.,** "Anti-oxidant, anti-inflamatory and anti-allergic activities of Luteolin" *Planta Med.*, 74(14), 1667-1677, **2008.**

10. **Adam, M., Dobias, P., Eisner, A., Ventura, K.,** "Extraction of antioxidants from plants using ultrasonic methods and their antioxidant capacity" *J. Sep. Sci.*, 32, 288-294, **2009.**

11. **Wang, H., Provan, G.J., Helliwell, K.,** "Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC" *Food Chem.*, 37, 307-311, **2004.**

12. **Petersen, M., Abdullah, Y., Benner, J., Eberle, D., Gehlen, K., Hücherig, S., Janiak, V., Kim, K.H., Sander, M., Weitzel, C., Wolters, S.,** "Evolution of rosmarinic acid biosynthesis" *Phytochemistry*, 70, 1663-1679, **2009.**