Original Article

TIPS Trends in Pharmaceutical Sciences 2020: 6(3): 205-212.

Quantification of Rosmarinic Acid and Caffeic Acid in Various *Salvia* Species by High Performance Thin Layer Chromatography

Forough Karami[1], Samira Jaberi[3], Afshin Afsari[3], Mahmoodreza Moein[3,4,*]

[1]Central Research Laboratory, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

[2]Department of Chemistry, Yasouj University, Yasouj, Iran.

[3]Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

[4]Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

Plants with high amounts of polyphenolic constituents may act as antioxidants or as producer of other mechanisms providing anticarcinogenic or cardioprotective actions. So, the objective of the present survey is quantitation of rosmarinic acid and caffeic acid as polyphenol compounds by means of high performance thin layer chromatography (HPTLC) in different *Salvia* species such as *Salvia santolinfolia* Boiss, *Salvia macilenta* Boiss, *Salvia compressa* Vent, *Salvia mirzayanii* Rech. f. & Esfand, and *Salvia sharifii* Rech. f. & Esfand. The plant materials were collected from Hormozgan Province and methanolic extracts of them were studied by HPTLC. An HPTLC plate silica gel 60 F_{254} as stationary phase, toluene-ethyl acetate-formic acid with ratio of 67.72: 22.90: 9.38, respectively as mobile phase were selected and quantitation of rosmarinic acid and caffeic acid were done at 366 nm. It was found that *S. compressa* (45.72±1.6 mg/g) and *S. mirzayanii* (464.64±0.028 mg/g) have higher amounts of rosmarinic acid and caffeic acid, respectively. Amounts of rosmarinic acid for other *Salvia* species were obtained as; *S. mirzayanii* 8.50±0.46, *S. santolinfolia* 7.34±0.15, *S. macilenta* 5.64±0.18, and *S. sharifii* 4.52±0.11 mg/g for each of dried plants. Similar analysis for caffeic acid was achieved as 11.32±2.16 for *S. santolinfolia*, 5.22±1.13 for *S. macilenta*, 1.93±1.19 for *S. compressa*, and for *S. sharifii* 4.63±3.26 mg/g for each of dried plants. In the developed method, *Salvia officinalis* was used as a standard module with amounts of 195.58 and 75.5 mg/g for rosmarinic acid and caffeic acid, respectively.

Keywords: Caffeic acid, Polyphenol, Rosmarinic acid, *Salvia*, High performance thin layer chromatography

1. Introduction

One of the most important genera Lamiaceae is *Salvia*, with antiseptics, astringents and spasmolytics properties (1) that comprises of about 900 species spread out the world, of which 56 species in the flora of Iran (2), 17 are endemic to Iran (3). Phenolic acids, phenolic glycosides, flavonoids, anthocyanins, coumarins, polysaccharides, sterols, terpenoids, and essential oils were detected in *Salvia* species (4, 5). Moreover, antioxidant, antimicrobial, and antiviral activities of some *Salvia* species has been reported (6, 7).

S. mirzayanii Rech. f. & Esfand (which is known Moor-e-Talkh, or "Marve-Talkh" in Persian) (8), classified as endemic species in the south of Iran, is used in the treatment of different ailments such as diabetes, spasms, gastrointestinal disorders, infections, and inflammations (9, 10). Antimicrobial activities of essential oil of *S. mirzayanii* leaves revealed that its essential oil inhibited the growth of standard and clinically isolat-

Corresponding Author: Mahmoodreza Moein, Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.
Email: mrmoein@sums.ac.ir

Recieved: 01/07/2020; Accepted: 01/09/2020

ed tested yeasts (11). 1,8-cineole (41.2%), linalool acetate (11.0%) and α-terpinyl acetate (6.0%) were identified as major compounds in essential oil of *S. mirzayanii* (11).

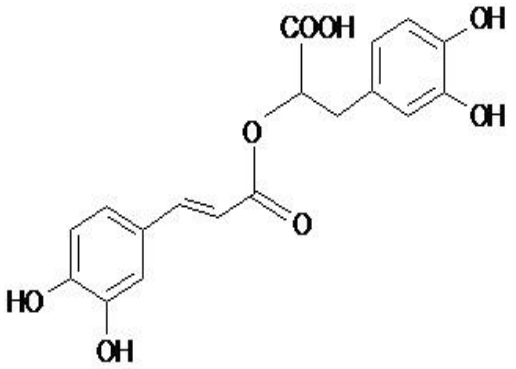
Another endemic *Salvia* species is *S. santolinifolia* Boiss that chemical composition analysis, antioxidant, antiglycating activities, and neuroprotective effects of that was reported by Asadi et al. (12). Also, antioxidant and neuroprotective properties of methanolic extracts of some *Salvia* species were investigated by Asadi *et al*. (13) and *S. macilenta* Boiss showed high amount of phenolic compounds. Khoramian Tusi and Khodagholi reported antiglycating activity and anti-apoptotic effects of *S. macilenta* (14).

Salvia sharifii Rech. f. & Esfand (which is known Maryam-goli-e-jonoobi in Persian) with antiseptic, carminative, digestive, and analgesic properties, as an endemic plant, in the south of Iran (15) was investigated in the current report. Farjam *et al*. studied about chemical composition and biological activties of the aerial parts of *S. sharifii* (15).

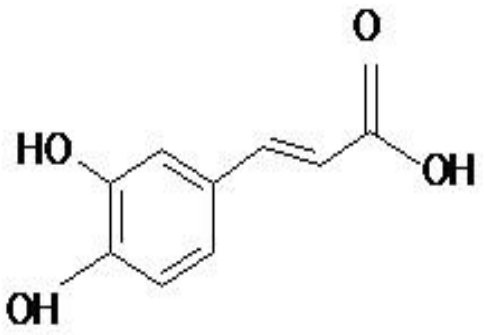
Polyphenols, as natural compounds, have benefit roles on human health due to the high antioxidant activity which rise from phenolic agents in their chemical structures (16). Attendance of numerous phenolic compounds in *Salvia* species belonging to the sorts of phenolic acids, phenolic glycosides, phenolic diterpenes, flavonoids, anthocyanins, and coumarins has been established (4, 17). Antioxidant activity of polyphenolic compounds has been described (18) and *in vitro* studies of polyphenols have been exposed to be more effective antioxidants than vitamins E and C (19, 20). Caffeic acid (CA), as a phenolic acid belongs to hydroxycinnamic acid derivatives, in sunflower seeds is the most phenolic acid (21) and significantly affects the solubility of plant proteins (22). In biochemistry of Lamiaceae family, CA shows a significant role and occurs mainly in the dimer form as rosmarinic acid (23).

An ester of CA and 3,4-dihydroxyphenyl lactic acid is rosmarinic acid (RA) with antiinflammatory, antimicrobial, antioxidant, immunomodulatory properties (24, 25) and its biosynthetic pathways was represented by Petersen *et al*. (26). For the first time in 1958, two chemists from Italy separated and purified RA from *Rosmarinus officinalis* and its name was chosen based on the plant that they separated it (27). Ellis and Towers in 1970 showed the backbone of RA is including two amino acids; L-tyrosine and L-phenylalanine (28).

Rich sources of polyphenols such as fruits, vegetables, whole grains, tea, chocolate, and wine (29) are significant in the food industries as they delay lipid peroxidation and cause to increase the quality and nutritional worth of products (30). Recently, high performance thin layer chromatography (HPTLC) as a modern technique is used for simultaneous analysis of various samples especially herbal extract with small amount of solvent and easy separation process even for two-dimensional separation. With regard to high amounts of flavonoid and phenolic acid in *Salvia* genus, antioxidant, and free radical scavenging activity of *Salvia*, we decided to determine RA and CA in some *Salvia* species by means of HPTLC as a simple,



Rosmarinic acid



Caffeic acid

Figure 1. Chemical structures of rosmarinic acid and caffeic acid.

rapid, reproducible, and inexpensive method.

2. Material and Methods

2.1. Chemicals

HPTLC plate silica gel 60 F_{254} (20×10 cm, Merck, Darmstadt, Germany); toluene; ethyl acetate (Samchun, Korea); formic acid with purity of 99.9% (Biochem, France); rosmarinic acid and caffeic acid (Sigma-Aldrich, Germany) were purchased. All materials were of analytical grade.

2.2. Sample Preparation

The plant materials were collected from Hormozgan Province in February 2015 and 2018. Geographical distribution and voucher specimens of *Salvia* species are presented in Table 1. Methanolic extracts were prepared as follows; after drying the plants, 1000 mg of each plant powders were dispersed in methanol, inserted in ultrasonic water bath at temperature of 80 °C for 15 min, filtered, and concentrated under reduced pressure on a rotary evaporator. The process was continued by addition of n-hexane, four times. Finally, methanolic phase was concentrated using a rotary evaporator and speed vacuum and diluted with methanol.

Standard solutions of RA and CA, 0.01 mg mL^{-1} in methanol were prepared.

2.3. Instrumental conditions

HPTLC analysis at room temperature and 20% humidity was done by Camag HPTLC, made in Switzerland, equipped with ATS4, ADC2, scanner 3 and visualizer. The samples, in the form of band, were spotted by ATS4 under N_2 gas (5 bar pressure) on a HPTLC plate silica gel 60 F_{254} (20 × 10 cm) with band length of 6 mm and distance of between tracks 11.3 mm. Developing of plate was done by ADC2 to the following parameters: toluene- ethyl acetate-formic acid with ratio of 67.72: 22.90: 9.38%, respectively as mobile phase; plate preconditioning time 1.0 min; filling volume 10 mL; migration distance 75 mm; and drying time 1.0 min.

Scanning of plates at the wavelength of 366 nm and absorption mode was carried out by scanner 3 to the following settings; slit dimension, 6.00 mm ×0.40 mm, macro; scanning speed, 20 mm/s; data resolution, 100 µm/step; and lamp D_2. Finally, the image of plates was obtained by visualizer at 254, 366 and visible wavelengths. Recording of data was done by WinCATS software.

3. Results

In the present study, gallic acid, coumaric acid, ferulic acid, quercetin, CA, and RA as phenolic compounds in *Salvia* species were investigated and finally the presence of CA and RA were established. The best solvent system for chromatographic separation was chosen based on previous report containing toluene-ethyl acetate-formic acid with the ratio of 67.72- 22.90 and 9.38%, respectively (31). The spots of RA and CA, seen as blue bonds on the HPTLC plate, were observed at an R_f value of 0.12±0.01 for RA and 0.27±0.01 for CA (Figure 2). Subsequently, there was no overlap with each other. TLC scanner 3 was used for detection and quantitation of RA and CA at the wavelength of

Table 1. Ecological distribution of *Salvia* species.

Name of plant	Herbarium No.	Location	Date	Voucher specimen
S. macilenta	MPPRC-93-1	Rosdan- Bandar abbas	February 2015	Hormozgan agriculture research center
S. santolinfolia	MPPRC-97-2	Hajjiabad-Hormozgan Province	February 2018	Hormozgan agriculture research center
S. compressa	MPPRC-94-4	Ghotb Abad Korea-Bandar abbas	February 2018	Hormozgan agriculture research center
S. mirzayanii	MPPRC-93-6	Tange Zagh mountain-Bandar abbas	February 2018	Hormozgan agriculture research center
S. sharifii	MPPRC-97-3	Fareghan-Hormozgan Province	February 2018	Hormozgan agriculture research center

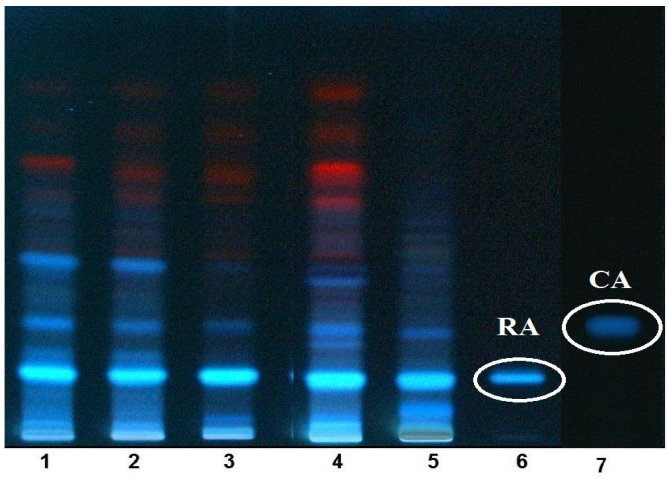


Figure 2. HPTLC chromatograms of *Salvia* extracts under 366 nm (1: *S. santolinfolia*, 2: *S. macilenta*, 3: *S. compressa*, 4: *S. mirzayanii*, 5: *S. sharifii*, 6: Rosmarinic acid and 7: caffeic acid).

366 nm. RA and CA with different R_f values were visualized as blue bonds under 366 nm.

Standard solutions of RA and CA 0.01 mg mL^{-1} in methanol was used to find calibration curves of RA at 7 concentrations of 70, 100, 120, 150, 200, 220 and 300 ng/spot and 85, 95, 105, 145, 185 and 225 ng/spot for CA by plotting the range of applied amounts of standard compounds versus corresponding area of each bond. Both of analyses were done in amount per fraction mode. In the established method, *S. officinalis* was used as a standard module with amounts of 195.58 and 75.5 mg/g for RA and CA, respectively.

In the following, accuracy and precision of procedure were investigated. The percentage of the systematic error is presented accuracy of method which is considered as the standardized agreement between the measured and the accurate value. Acceptable values for accuracy and precision assay at all concentrations should be ±15% (32). Method accuracy of RA and CA was calculated between -0.48-6.59% and -3.83-13.5%, respectively. The percentage coefficient of variation (CV%) for replicate assays is defined the method precision. Intra-day analysis was done by repeated concentrations in triplicates on the same day but inter day was measured as analysis of three repeated concentrations on three various days (Tables 2 & 3). The intra-day and inter-day precision were obtained in the range of 1.34-4.55% for RA and 1.6-6.54% for CA.

4. Discussion and conclusion

In the current research, determination of RA and CA was performed via HPTLC technique as an efficient, quick, reproducible, precise, accurate, and simple method. After chromatographic separation, visualization of phenolic compounds was carried out at the wavelength of 366 nm and without using specific indicator.

Polynomial regression analysis caused to a calibration curve with the equation of $y=-0.021\ x^{\wedge 2}+21.885\ x+837.73$ and $r^2=0.9976$ for RA and $y=-0.0078\ x^{\wedge 2}+7.3889\ x-259.38$ for CA with $r^2=0.9906$. High correlation of measurement (r^2) values present high correlation of the fitted regression lines. A range of 4.52-45.72 mg/g and 1.93-464.64 mg/g was obtained for RA and

Table 2. Precision and accuracy results at inter-day and intra-day for rosmarinic acid.

Amount	Intra-day		Inter-day	
(ng/spot)	Accuracy%	Precision%	Accuracy%	Precision%
50	6.59	4.32	3.96	2.31
170	1.04	4.06	-0.48	1.34
250	0.01	4.55	1.54	1.44

Table 3. Precision and accuracy results at inter-day and intra-day for rosmarinic acid.

Amount	Intra-day		Inter-day	
(ng/spot)	Accuracy%	Precision%	Accuracy%	Precision%
125	13.5	1.6	-1.2	2.62
165	4.60	1.94	-1.10	4.39
205	12.8	5.35	-3.83	6.54

CA in investigated *Salvia* extracts, in that order given. It was identified that *S. compressa* showed maximum and minimum amounts of RA and CA, respectively. On the other hand, for extracts of *S. macilenta* and *S. sharifii*, contents of RA and CA were almost the same. For *S. santolinfolia*, the amount of CA was higher than RA. Although, the highest amount of CA was observed in *S. mirzayanii*, the value of RA was less than *S. compressa*. Comparison of data with *S. officinalis* as a standard module indicated that amounts of RA and CA (exception of *S. mirzayanii*) were lower than *S. officinalis* contents. Amounts of RA and CA for presented *Salvia* extracts based on mg/g for each of dried plants and distribution of two phenolic acids for the mentioned plants were reported in Table 4 and Figure 3, respectively.

Table 4. The contents of rosmarinic acid and caffeic acid in methanolic extracts of different *Salvia* species.

Name of plant	Rosmarinic acid		Caffeic acid	
	Found (mg/g±SD, n=9)	CV%	Found (mg/g±SD, n=9)	CV%
S. santolinfolia	7.34±0.15	2.10	11.32±2.16	1.90
S. macilenta	5.64±0.18	3.22	5.22±1.13	1.08
S. compressa	45.72±1.6	3.46	1.93±1.19	1.55
S. mirzayanii	8.50±0.46	5.36	464.64±0.028	1.02
S. sharifii	4.52±0.11	2.49	4.63±3.26	6.75

In conclusion, analysis of data at inter-day and intra-day showed that the mentioned method has a good accuracy and precision for RA and CA measurements. Also, the method is assumed to be ideally appropriate for quick and routine analyses. The achieved findings can help to assess pharmacological activity of the desired plants and regard

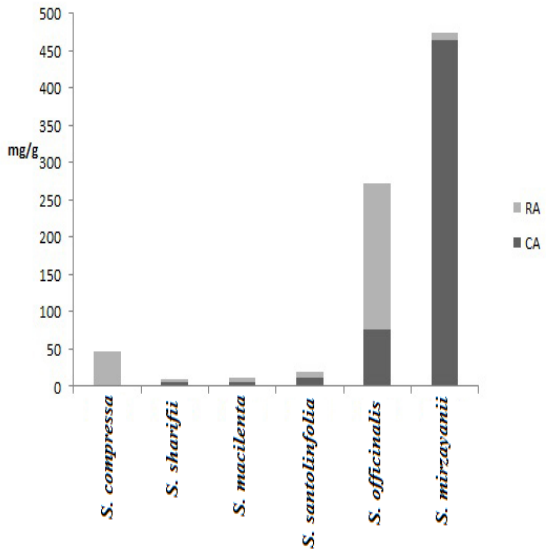


Figure 3. Distribution amounts of rosmarinic acid and caffeic acid in studied *Salvia* species.

to high amounts of CA and RA in *S. mirzayanii* and *S. compressa*, respectively, they show the highest antioxidant effects. The observed different amounts of phenolic acids in *Salvia* extracts may be related to environmental conditions, genetic of the plants, and the extraction method.

Acknowledgments

The authors wish to thank the support of Shiraz University of Medical Sciences, Shiraz, Iran for providing financial budget of the of presented research (Grant number 18113).

Conflict of Interest

None declared.

References

1. Newall CA, Anderson LA, Phillipson JD: Herbal medicines. A guide for health-care professionals. The pharmaceutical press, London, 1996; pp. 231
2. Naghibi F, Mosaddegh M, Mohammadi Motamed M, Ghorbani A. Labiatae family in folk medicine in Iran: from ethnobotany to pharmacology. *Iran J Pharm Res*. 2010;4(2):63-79.
3. Mozafarian V: A dictionary of iranian plant names (Latin English Persian). Tehran, Iran, Farhang Mosafer Publication, 1996; pp. 207
4. Lu Y, Foo LY. Polyphenolics of Salvia--a review. *Phytochemistry*. 2002 Jan;59(2):117-40. doi: 10.1016/s0031-9422(01)00415-0. PMID: 11809447.
5. Ghannadi A, Samsam-Shariat SH, Moattar F. Volatile constituents of the flower of Salvia hydrangea DC. Ex. Benth. *Daru*. 1999;7(3):23–5.
6. Sivropoulou A, Nikolaou C, Papanikolaou E, Kokkini S, Lanaras T, Arsenakis M. Antimicrobial, cytotoxic, and antiviral activities of Salvia fructicosa essential oil. *J Agric Food Chem.* 1997;45(8):3197-201. doi:10.1021/jf970031m.
7. Li L-N. Biologically active components from traditional Chinese medicines. *Pure & Appl Chem*. 1998;70(3):547-54.
8. Sonboli A, Babakhani B, Mehrabian AR. Antimicrobial activity of six constituents of essential oil from Salvia. *Z Naturforsch C J Biosci*. 2006 Mar-Apr;61(3-4):160-4. doi: 10.1515/znc-2006-3-401. PMID: 16729570.
9. AAmirghofran Z, Bahmani M, Azadmehr A, Javidnia K, Ramazani M, Ziaei A. Effect of Salvia mirzayanii on the immune system and induction of apoptosis in peripheral blood lymphocytes. *Nat Prod Res.* 2010 Apr;24(6):500-8. doi: 10.1080/14786410802267502. PMID: 20397102.
10. Rowshan V, Tarakemeh A. Comparison of Salvia mirzayanii volatile compounds extracted by headspace extraction and hydrodistillation methods. *Int J Plant Anim Environ Sci*. 2013;3:136-40.
11. Zomorodian K, Moein M, Pakshir K, Karami F, Sabahi Z. Chemical Composition and Antimicrobial Activities of the Essential Oil From Salvia mirzayanii Leaves. *J Evid Based Complementary Altern Med*. 2017 Oct;22(4):770-776. doi: 10.1177/2156587217717414. Epub 2017 Jul 10. PMID: 28689440; PMCID: PMC5871294.
12. Asadi S, Khodagholi F, Esmaeili MA, Tusi SK, Ansari N, Shaerzadeh F, Sonboli A, Ghahremanzamaneh M. Chemical composition analysis, antioxidant, antiglycating activities and neuroprotective effects of S. choloroleuca, S. mirzayanii and S. santolinifolia from Iran. A*m J Chin Med*. 2011;39(3):615-38. doi: 10.1142/S0192415X1100907X. PMID: 21598426.
13. Asadi S, Ahmadiani A, Esmaeili MA, Sonboli A, Ansari N, Khodagholi F. In vitro antioxidant activities and an investigation of neuroprotection by six Salvia species from Iran: a comparative study. *Food Chem Toxicol*. 2010 May;48(5):1341-9. doi: 10.1016/j.fct.2010.02.035. Epub 2010 Mar 1. PMID: 20197079.
14. Tusi SK, Khodagholi F. Salvia macilenta exhibits antiglycating activity and protects PC12 cells against H2O 2-induced apoptosis. *Cytotechnology.* 2014 Jan;66(1):169-79. doi: 10.1007/s10616-013-9550-x. Epub 2013 Apr 12. PMID: 23579246; PMCID: PMC3886546.
15. Farjam MH, Rustaiyan A, Ezzatzadeh E, Jassbi AR. Labdane-Type Diterpene and Two Flavones from Salvia Sharifii Rech. f. and Esfan. and their Biological Activities. *Iran J Pharm Res.* 2013;12(2):395-400.
16. David IG, Bizgan AM, Popa DE, Buleandra M, Moldovan Z, Badea IA, Tekiner TA, Basaga H, Ciucu AA. Rapid determination of total polyphenolic content in tea samples based on caffeic acid voltammetric behaviour on a disposable graphite electrode. *Food Chem.* 2015 Apr 15;173:1059-65. doi: 10.1016/j.foodchem.2014.10.139. Epub 2014

Nov 3. PMID: 25466125.
17. Cuvelier ME, Berset C, Richard H. Antioxidant constituents in sage (Salvia officinalis). *J Agri Food Chem.* 1994;42:665-9. https://doi: 10.1021/jf00039a012.
18. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M. Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem.* 1999 Oct;47(10):3954-62. doi: 10.1021/jf9901461. PMID: 10552749.
19. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med.* 1996;20(7):933-56. https://doi: 10.1016/0891-5849(95)02227-9.
20. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic Res.* 1995 Apr;22(4):375-83. doi: 10.3109/10715769509145649. PMID: 7633567.
21. Leung J, Fenton TW, Clandinin DR. Phenolic components of sunflower flour. *J Food Sci.* 1981;46(5):1386-8. doi: 10.1111/j.1365-2621.1981.tb04180.x.
22. Mieth G, Pohl J, Kozlowska H, Rotkiewicz DA. Phenolic constituents of sunflower seed II. Effect of phenolic acids on solubility characteristics of plant proteins. Nahrung. 1992;26:K11-K3.
23. Gerhardt U, Schroeter A. Rosmarinic acid-a naturally occurring antioxidant in spices. Fleischwirtschaft. 1983;63:1628-30.
24. Baxter H, Harborne JB, Moss GP: Phytochemical dictionary: a handbook of bioactive compounds from plants. CRC press, 1998.
25. Jean B. Pharmacognosy, phytochemistry, medicinal plants (4th ed.). Lavoisier; 2009 Oct 2.
26. Petersen M, Abdullah Y, Benner J, Eberle D, Gehlen K, Hücherig S, et al. Evolution of rosmarinic acid biosynthesis. *Phytochemistry.* 2009;70(15):1663-79. https://doi: 10.1016/j.phytochem.2009.05.010.
27. Scarpati ML, Oriente G. Isolamento e costituzione dell'acido rosmarinico (dal rosmarinus off.). Ric Sci. 1958;28:2329-33.
28. Ellis BE, Towers GH. Biogenesis of rosmarinic acid in Mentha. *Biochem J*. 1970;118(2):291-297. doi:10.1042/bj1180291
29. Tsao R. Chemistry and biochemistry of dietary polyphenols. *Nutrients*. 2010;2(12):1231-1246. doi:10.3390/nu2121231
30. Wojdyło A, Oszmiański J, Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chem.* 2007;105(3):940-9. https://doi: 10.1016/j.foodchem.2007.04.038.
31. Jaberi S, Moein M, Jafari A, Karami F. Detection and quantification of some chemical compositions of Thymus daenensis and Thymus lancifolius by HPTLC. International Symposium for High-Performance Thin-Layer Chromatography, 4 – 8, July 2017, Berlin2017. p. 78.
32. Campanero M, Zamarreno A, Diaz M, Dios-Vieitez M, Azanza J. Development and validation of an HPLC method for determination of amphotericin B in plasma and sputum involving solid phase extraction. *Chromatographia*. 1997;46(11-12):641-6. https://doi: 10.1007/BF02490525.