QUANTITATIVE ANALYSIS OF PHENOLIC COMPOUNDS FROM *SALVIA OFFICINALIS* L. LEAVES

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Abstract

The aim of this work was to investigate changes in total phenolic, flavonoids and phenolcarboxylic acids (chlorogenic, caffeic, rosmarinic) contents of indigenous sage (*Salvia officinalis* L.) leaves collected monthly (from May to September) and their correlation with climate conditions. Our results showed that variation in total phenolics amount can be explained by changes in environmental factors (such as temperature and humidity). The highest content of rosmarinic acid was detected in May (1.0560 g%), July (1.4270 g%) and September (1.3325 g%). Total polyphenols, phenolcarboxylic acids and flavonoids contents showed the same trend, a constant increase being recorded from May to July (3.2698±0.964 g%, 2.4041±0.0964 g%, 1.4415±0.040 g% for May and 6.3297±0.2042 g%, 5.4916±0.2273 g%, 2.0994±0.0944 g% respectively).

Rezumat

Cercetările au urmărit determinarea cantitativă a unor derivaţi polifenolici (flavone, acid rozmarinic, acid cafeic, acid clorogenic, polifenoli totali) din frunzele de salvie (*Salviae folium*), cultură indigenă, recoltate în anumite perioade ale anului (lunile mai-septembrie), în scopul determinării dinamicii de acumulare a acestora dependent de condiţiile climatologice.

Rezultatele obţinute au evidenţiat faptul că biosinteza derivaţilor polifenolici este dependentă de factorii climatologici (umiditate şi temperatură); cel mai crescut conţinut în acid rozmarinic s-a înregistrat în lunile mai (1,0560 g%), iulie (1,4270 g%) şi septembrie (1,3325 g%), în acid clorogenic în toate probele analizate, concentraţia variind între 2,4041 ±0,0964 (luna mai) şi 5,4916±0,2273 g% (luna iulie); conţinutul în derivaţi flavonici este cuprins între 1,4415 ±0,040 (luna mai) şi 2,0994±0,0944 g% (luna iulie) iar în polifenoli totali între 3,2698 ±0,964 (luna mai) şi 6,3297±0,2042 g% (luna iulie).

Keywords: *Salviae folium*, phenolcarboxylic acids, thin layer chromatography, HPLC.

Introduction

Leaves of *Salvia officinalis* L. (Lamiaceae) are used for both culinary and therapeutic purposes. They possess hypoglycemiant, spasmolytic, stomahic, estrogenic choleretic [4, 9, 14, 22, 25], antioxidant,

antiproliferative [11], anti-inflammatory [1] and gastroprotective properties [15]. The phytochemical constituents of sage leaves include: flavonoids (luteolin, apigenin, quercetin-glycosides) [18], phenolcarboxylic acids (caffeic, chlorogenic, rosmarinic, ferulic), volatile oil (monoterpenic derivates), tannins, carnosol [15], bittter and triterpenic substances [4, 9, 14, 15, 22, 25].

Among phenolic compounds, caffeic and chlorogenic acids are well known for their hepatoprotective and hypolipidaemic activity, through inhibition of lipid peroxidation and antioxidant properties [10]. Rosmarinic acid (2-hydroxi-dihydrocaffeic acid) has a wide spectrum of biological activities including anti-inflammatory, anti-microbial, antioxidant and immunomodulatory properties [3, 6]. Moreover, rosmarinic acid and caffeic acid have antidepressant and anxiolytic effects, through inhibition of monoaminoxidase activity and modulatory properties upon α_1- and α_2-adrenergic receptors [23, 24].

Taking into consideration the data from scientific literature [5] regarding the biosynthesis of phenolic compounds in *Lamiaceae* species, the aim of our study was to investigate the correlation between environmental factors (humidity, temperature) and phenolic compounds accumulation in sage leaves harvested at different stages of development (from May to September).

Materials and Methods

Plant material. Leaves of *Salvia officinalis* L. (sage) were harvested from Radu-Vodă village, Călăraşi county (geographic coordonates 44°23'11" N, 26°55'35"E) [8], Romania, on one day, between the 15th and the 20th of May (SM), June (SI), July (SIL), August (SA) and September (SS) 2013. Leaves were air-dried in the shade, at room temperature. Herbarium voucher samples are deposited in the Department of Pharmacognosy, Faculty of Pharmacy, ”Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania.

Reagents and solvents. All reagents and solvents were from Roth (Germany), unless otherwise stated. HPLC solvents, diphenylboriloxyethylendiamine (DFBOA) were provided by Sigma-Aldrich (Germany) and thin layer chromatography (TLC) plates were purchased from Merck (Germany).

For polyphenols analysis, qualitative (TLC) and quantitative (spectrophotometric and HPLC) methods have been used.

Preparation of plant extracts. For TLC, spectrophotometric and HPLC analysis, 5 g of raw material (batches SM, SI, SIL, SA and SS) were

heated twice with 70% ethanol on a reflux condenser for 30 and 15 min. respectively. Additionally for the HPLC determination, 10 mL of 100g/L HCl were added to 10 mL of 70% ethanolic solutions and the mixture was heated on a reflux condenser for 40 min.

Thin layer chromatography (TLC) was performed using an aluminium coated TLC plate (20 x 20cm, kept for 1h at 100°C before use) and ethylacetate : formic acid : water = 90 : 6 : 6 (v/v/v) as eluent system. Plates were spotted with 70% ethanolic sample solutions (SM, SI, SIL, SA, SS) and 0.1 mg/mL methanolic references solutions (rutin, hyperoside, isoquercitrin, caffeic acid, chlorogenic acid and rosmarinic acid). The plate was developed over a path of 8 cm, air-dried, sprayed with a 5g/L solution of DFBOA (in ethylacetate) and heated at 100°C (5 min.) for optimal colour development. The plate was examined in UV light (λ = 365 nm) using a Camag Reprostar Lamp with Epson Photo PC850, before and after spraying with the detection reagent [16, 27].

Spectrophotometric determination of total polyphenols (expressed as tannic acid equivalent) was performed with *Folin-Ciocâlteu* reagent according to Singleton & Rossi method [21] modified by Makkar *et* al. [13]. Flavonoid and phenolcarboxylic acids (AFC) contents (expressed as rutin and chlorogenic acid equivalents) were estimated based on the chelating reaction with aluminium chloride and formation of oxymes with *Arnow* reagent, respectively [27]. For all determinations a Jasco V-530 (Jasco, Japan) spectrophotometer was used. Calibration curves of tannic acid (linearity range: 2.04-9.18 µg/mL, R^2 = 0.9994, n = 8), rutin (linearity range: 5-35 µg/mL, R^2 = 0.9997, n = 11) and chlorogenic acid (linearity range: 0.0113-0.0527 mg/mL, R^2 = 0.9998, n = 6) were used to calculate the active substances contents.

HPLC analysis was carried out using a Jasco HPLC MD-2015 equipped with degasser, binary gradient pump, column thermostat and UV detector. The separation was achieved on a reverse-phased analytical column (Nucleosil – C18, 25 x 0.4mm i.d., 5µm particle). The mobile phase consisted of a mixture of water and phosphoric acid = 999 : 1 (v/v) (solvent A) and acetonitrile (solvent B). The gradient used was: 90%A/10%B, 0 min.; 90 → 78 A/10 → 22/B, 0-13 min; 78 → 60A/ 22 → 40/B, 13-14 min.; 60%A/40%B, 14-20 min. The flow rate was 1.5 mL/min and the injection volume 20 µL. The monitoring wavelength was 310 nm. The analytical data was evaluated using a Jasco data processing system (Chrompass). Methanolic solutions of chlorogenic, caffeic, rosmarinic, ferulic acids, quercetin, kaempferol and rutin were used as standards (Table I, Fig. 1). Calibration curves in the 4.06-370 µg/mL range had a good linearity (R^2 > 0.99,

n = 5). Phenolic compounds from samples were identified based on their chromatographic retention times and UV spectra and quantified by comparing integrated peak areas to calibration curves prepared with the mentioned standards.

Table I
Retention time for standards

Compound	Retention time (min.)
Chlorogenic acid	7.122
Caffeic acid	7.960
Ferulic acid	13.145
Rutin	15.198
Quercetin	18.872
Kaempferol	20.25
Rosmarinic acid	17.58

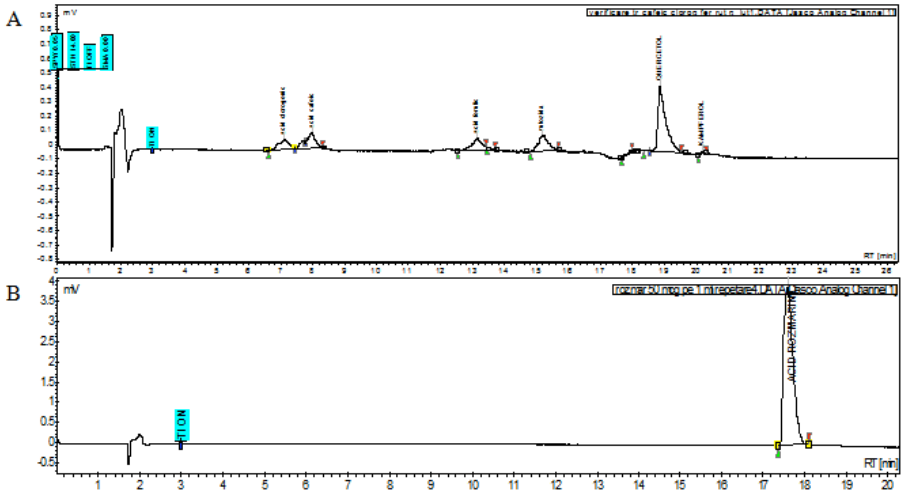


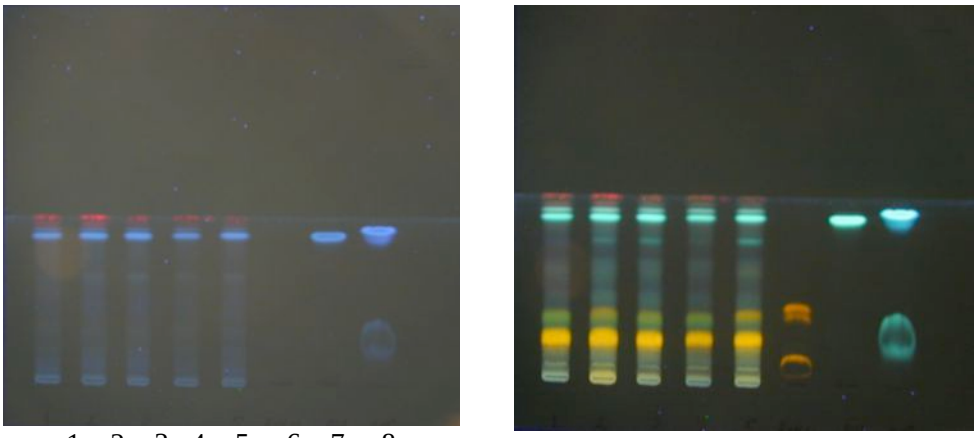
Figure 1
Chromatograms of standards (A – mixture of chlorogenic acid, caffeic acid, ferulic acid, quercetin, kaempferol and rutin; B – rosmarinic acid)

Statistical analysis. The data analysis was performed using Microsoft Excel 2007 software. The spectrophotometric determinations represent the average ± standard deviation (SD) of three independent replicates.

Results and Discussion

TLC analysis of polyphenols showed the presence of spots (in all batches) with the same R_f (retention factor) value as rosmarinic acid (R_f = 0.88) and caffeic acid (R_f = 0.93) (Fig. 2). Hyperoside (R_f = 0.37) was identified

only in SIL and SS samples. Analysing the chromatograms, one can note the presence of other spots corresponding to compounds with flavonoidic behaviour (brown fluorescent zones before spraying with DFBOA reagent and yellow ones after) or to phenolcarboxylic acids (blue fluorescent zones before spraying with DFBOA and green-blue/light blue ones after), that were not identified due to the lack of standards. Red fluorescent spots correspond to chlorophyll.



Legend: 1 – SM, 2 – SI, 3 - SIL, 4 - SA, 5 - SS, 6 – rutin + hyperoside +isoquercitrin (from downword to top), 7 – rosmarinic acid, 8 – chlorogenic acid + caffeic acid (from downword to top)

Figure 2
TLC chromatogram of samples solutions
(A – before spraying with DFBOA, B – after spraying with DFBOA)

Spectrophotometric and HPLC results are presented in Table II, III.

Table II
Spectrophotometric determination of sage polyphenols

Batch	AFC (g% chlorogenic acid)	Flavonoids (g% rutin)	Total polyphenols (g% tannic acid)
SM	2.4041 ±0.0964	1.4415 ±0.040	3.2698 ±0.964
SI	4.3320 ±0.077	1.6228±0.0175	4.8184 ± 0.7238
SIL	5.4916±0.2273	2.0994±0.0944	6.3297±0.2042
SA	3.1245±0.0364	1.1323 ±0.0319	3.8906±0.1592
SS	4.9779±0.1217	2.0775±0.2545	5.7054±0.1488

Table III

HPLC determination of sage polyphenols

Batch	70% ethanolic solution		Hydrolised 70% ethanolic solution	
	g% caffeic acid	g% rosmarinic acid	g% caffeic acid	g% rosmarinic acid
SM	0.0257	1.0560	+	-
SI	-	0.6299	-	-
SIL	-	1.4270	0.0015	0.547
SA	-	0.4919	+	0.2643
SS	-	1.3325	-	0.4551

Legend: „-" not detected, „+" – detected, but under the limit of quantification

According to our spectrophotometric results (table II), sage leaves are a considerable source of phenolic compounds, the highest content of total polyphenols, flavonoids and AFC being observed for leaves collected in July (SIL). However, the polyphenols content is low compared to Proestos C. *et* al. (2005) and Roby M.H.H. *et* al. (2013) results, that found 0.595-1.36 g% polyphenols expressed as gallic acid (equivalent to 5.95-13.6 g% tannic acid) [17, 18].

Meteorological conditions (such as temperature and rainfall) could be responsible for large variations in phenolic contents. As discussed above, a high amount of polyphenols was recorded in July, a month characterized by high temperatures and few rainfalls. The same trend of polyphenols accumulation (under high temperatures and water deficit) was observed by Bettaieb I. *et.*al. [2] for aerial parts of *Salvia officinalis* L. and by Rodriguez A.S. *et* al. [19], for white and red onion. In June and September (rainy months), polyphenols content was relatively high, but low compared with July. It is well known that phenolic compounds have an important role in the resistance of plants to environmental stress and particularly water deficit. Under this constraint there is a constant increase in the formation of ROS (reactive oxygen species). Thus high temperatures and water deficit induce the protective mechanisms, involving the synthesis of phenolic compounds and subsequently the neutralization of ROS [2].

Regarding HPLC determinations (Table III), rosmarinic acid and caffeic acid contents in May was low compared with Vladimir-Knežević *et* al. [26] results that found 1.872 g% rosmarinic acid and 0.08 g% caffeic acid in sage leaves harvested before flowering [26]. Analysing the data, one can note major differences among rosmarinic acid content in different months that can be attributed to climate (average temperatures and rainfall). According to Romanian National Institute for Weather, the month of June 2013 was characterized by heavy rainfall (151-175 mm, with 25% deviation

from annual media [7]) and is associated with a low amount of rosmarinic acid (0.6229 g%)present in the rage leaves. Our results are similar to other authors [2] that found an inverse correlation between water amount and biosynthesis of AFC. The highest content of rosmarinic acid (1.4270 g%) was recorded in July (Table III, fig. 3). Although, the month of August 2013 was characterized by average temperatures above 24°C (25% deviation from annual media), rosmarinic acid content was low compared with July (0.4919 g%). An increase in rosmarinic acid amount was observed in September (1.3325 g%), although the average rainfall was high (126-150 mm, with a 300% deviation from annual media). It is possible that normal temperatures (16-18°C with a deviation of 0.5% from annual media) enhanced significantly the biosynthesis of phenolic acids.

Rosmarinic acid was also identified in SIL (Fig. 3), SA and SS hydrolysed solutions (Table III), so we concluded that it exists in both free, glycolised or esterified forms, which is in agreement with scientific literature [26, 27]. Its content is low compared to unhydrolysed solutions, probably due to chemical decomposition.

Although scientific literature foresees the presence of rutin, chlorogenic acid and ferulic acid in sage leaves [26], these compounds were not identified by us.

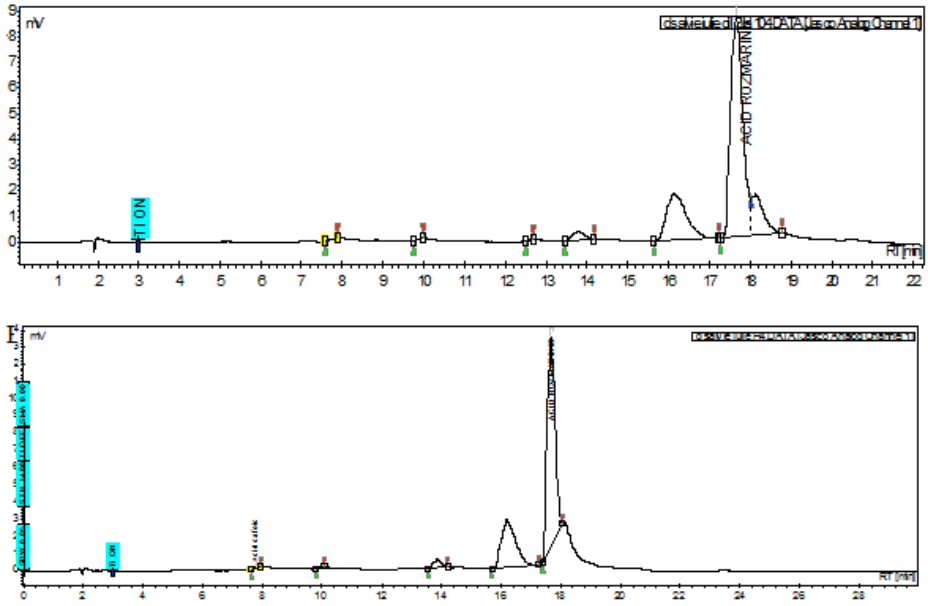


Figure 3
HPLC chromatograms for SIL (A – 70% ethanolic solution,
B – hydrolysed 70% ethanolic solution)

Conclusions

Salvia officinalis L. leaves are a considerable source of polyphenols (phenolcarboxylic acids, flavonoids). Using TLC and HPLC analysis we have identified and quantified rosmarinic and caffeic acids.

Based on our results, environmental factors, such as average temperatures and rainfall, have a key role in the biosynthesis of phenolic compounds in sage leaves.

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