CHEMICAL PROFILING OF POLYPHENOLS FROM *SALVIA OFFICINALIS* AND *THYMUS SERPYLLUM* EXTRACTS DURING A THREE-STAGE EXTRACTION PROCESS

Mioara PRUNDEANU[1], Ana-Maria BREZOIU[1], Mihaela DEACONU[1], Daniela BERGER[1]

The ethanol-water (4/1 v/v) extract fractions from Salvia officinalis L. and ethanol-water (4/1 v/v) and ethanolic extract fractions from Thymus serpyllum L. were prepared at 80 °C by conventional method using 1/10 g/mL vegetal material/solvent ratio. The aim of this study was to evaluate the phenolic compounds distribution during three-stage extraction by reverse-phase HPLC-PDA, The extract fractions were characterized by spectrometric determinations as follows: total polyphenols, flavonoids, total chlorophyll pigments content, as well as their antioxidant activity by both DPPH and ABTS methods. Surprisingly, in the first stage of extraction it was not always obtained the highest amount of polyphenolic compounds, so it can be concluded that three-stage extraction is an efficient process that leads to extracts with a high phytochemicals content and good radical scavenger capacity.

Keywords: polyphenolic extract; common sage extract; wilde thyme extract; HPLC-PDA

1. Introduction

Common sage (*Salvia officinalis* L.) and wilde thyme (*Thymus serpyllum* L.) are aromatic herbs that belong to the Lamiaceae family having high amounts of essential oils and polyphenolic compounds [1-3].

A high polyphenolic content is important considering studies related to these compounds with significant antioxidant or antimicrobial properties [1, 3-6]. Moreover, it was already proven that extracts obtained from various medicinal herbs present antioxidant, antimicrobial, anti-inflammatory or even antitumoral properties and their benefits for human health are well-known [7-10].

Salvia officinalis extracts present antioxidant properties which could be attributed to caffeic acid derivates as rosmarinic acid and flavones (glycosides of apigenin and luteolin) content. Also, several studies revealed that sage extracts [8, 11-12] or essential oil [3,13] exhibited antioxidant, antifungal, antitumor, antiallergic properties [14]. A recent study emphasized additional utilization of aqueous or ethanolic extracts from *Salvia officinalis*, being effective on the

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improvement of memory and cognitive functions proved in clinical trial of patients with Alzheimer or dementia, as well as through *in vivo* tests on rats, and on metabolic effects regarding the profiles of glucose and lipids [15].

Thymus serpyllum is also an important source of phytochemicals exhibiting antioxidant, antimicrobial, antitumor, and cytotoxic properties according to several studies [16-20]. Due to its pharmacological properties, the essential oil of wilde thyme represents an important ingredient used in the pharmaceutical products, supplements or as a functional ingredient in food products [19]. Regarding the antitumor activity of *Thymus serpyllum* extracts, a study showed that rosmarinic acid would be efficient for breast cancer treatment (MCF-7 cells resistant to Adriamycin), reducing cell viability with 14% when a concentration of 1.25 mM was applied [18].

Another key factor in preparing polyphenolic extracts from medicinal plants is the extraction method. For instance, the conventional extraction method is usually based on selecting the proper solvent and temperature in order to increase the solubility of targeted compounds. The polarity of the solvent plays an important role in the extraction of phenolic compounds. It has shown that alcohols, as well as alcohol-water mixtures are more environmentally friendly solvents [20]. Water and ethanol are from the toxicological point of view, much safer and more suitable than other solvents for the food and pharmaceutical industry [21]. However, recently were reported extraction procedures with enhanced yields based on ultrasounds [22] or microwaves for auxiliary energy [1], as well as supercritical CO_2 as solvent. For example, supercritical carbon dioxide extraction applied on *Salvia officinalis* lead to the obtaining of new phytocomponents with new therapeutic effects, especially for neurodegenerative diseases and various carcinomas [23].

For preparing wild thyme extracts with enhanced amount of polyphenols, modern techniques were also applied. For example, an increased polyphenolic content was achieved for hydro-ethanolic extracts obtained using ultrasound-assisted extraction, for a ground starting material with a particle size of 0.3 mm and plant/solvent ratio of 1/30. This study was reported to be an initial step in production of polyphenols-rich wild thyme extracts aimed to be used in formulation of foodstuffs and medicines [24]. Another study emphasized that subcritical water extraction led to the obtaining of an extract with enhanced amount of bioactive compounds, having very good antioxidant activity [25].

We previously reported high amount of polyphenols for ethanol and ethanol-water extracts prepared by conventional or microwave-assisted extraction from both common sage and wilde thyme that showed a good antimicrobial activity, especially the ethanolic common sage extract prepared by MW-irradiation [1]. This study emphasized the polyphenolic compounds distribution in each stage of the extraction process using ethanol or ethanol-water mixture from

both common sage and wild thyme. The chemical profile from each stage of the extraction through HPLC-PDA, as well as radical scavenger capacity by *in vitro* ABTS and DPPH methods were determined.

2. Experimental

2.1. Materials

All the reagents, potassium persulphate ($K_2S_2O_8$), ethanol (Sigma-Aldrich) and Folin-Ciocalteu reagent were used as received. For chromatographic analyses, the following standard HPLC-grade compounds were used: protocatechuic acid (TCI, >98%, HPLC-grade), caftaric acid (Molekula GmbH), caffeic acid (Sigma, 98%, HPLC-grade), chlorogenic acid (HWI group, primary reference standard), rosmarinic acid (Sigma, >98%, HPLC-grade), gallic acid (Alfa Aesar, 98%), catechin hydrate (Sigma, >98%, HPLC-grade), ellagic acid dihydrate (TCI, >98%, HPLC-grade), chicoric acid (TCI, >98%), *trans*-ferulic acid (TCI, >98%, GC), vanillic acid (TCI, >98%, GC-grade), syringic acid (Molekula, >98.5%), (−) epicatechin (TCI, >98%, HPLC-grade), quercetin (Sigma, >95%, HPLC-grade), rutin hydrate (Sigma, 95%, HPLC-grade), *trans*-*p*-coumaric acid (Sigma Aldrich, analytical standard), myricetin (Sigma, >96%, HPLC-grade), *trans*-resveratrol (Sigma Aldrich, certified reference material), kaempferol (Sigma, >97%, HPLC-grade), cyanidin chloride (Sigma, >95%, HPLC-grade), malvidin chloride (Sigma Aldrich, >95%, HPLC), pelargonidin chloride (Aldrich) and delphinidin chloride (Sigma Aldrich, analytical standard), solvents acetonitrile (ACN), formic acid, and for radical scavenger activity determination, 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich, 97%) and 2,2'-azino-bis (3 ethylbenzothiazoline-6-sulphonic acid) (ABTS, Sigma Aldrich).

Dried leaves of *Salvia officinalis* or *Thymus serpyllum* were purchased from local vendors and ultrapure water (Millipore Direct- Q3 UV water purification system with Biopack UF cartridge) was used for all solutions and experiments.

2.2. Preparation of polyphenolic extracts from Salvia officinalis and Thymus serpyllum

In all cases, the first step of the extraction process, a maceration of vegetal material in used solvent for 16-20 h was carried out, in order to enhance the polyphenolic compounds recovery. The hydroalcoholic (ethanol/water=4/1 v/v) conventional extracts from both plants were prepared at reflux, in three extraction stages of 1 h with the separation of the vegetal material after each step (vegetal material/solvent=1/10 g/mL), the replacement of the solvent in the same volume and then the extracts were stored and analysed separately. These extracts were denoted So and Ts for common sage and *Thymus serpyllum*, respectively,

followed by the fraction number (i.e. So-FI). In the case of *Thymus serpyllum*, it was performed a conventional extraction using only ethanol in the same conditions as for the hydro-alcoholic extract and it was denoted Ts(EtOH) followed by the extract fraction number.

2.3. Characterization of phenolic extracts

Polyphenolic extracts were characterized by several spectrophotometric methods (Shimadzu UV-1800) to determine total polyphenols, flavonoids and total chlorophyll contents, while the extract composition was assessed using reversed-phase high performance liquid chromatography with photodiode array detector, HPLC-PDA (Shimadzu Nexera 2). The description of the spectrophotometric methods and HPLC analysis were previously reported in our paper [1, 4].

The radical scavenger activity (RSA) of polyphenolic extracts was assessed by both DPPH and ABTS assays. The detailed procedures for each determination are presented elsewhere [4].

3. Results and discussion

3.1. Spectrophotometric characterization of polyphenolic extracts

Polyphenolic extracts have attracted an increased interest due to their beneficial properties such as antioxidant or antimicrobial activity. However, polyphenolic extracts composition is highly dependent on the extraction conditions, vegetal material, as well as storage conditions. This study describes the chemical profiling of polyphenolic extracts during a three-stage extraction process and how the antioxidant activity depends on the solvent.

The amount of polyphenols extracted in each extraction stage for all extracts are presented in Figure 1. As it can be observed the highest quantity of polyphenols is usually extracted in the first stage, while second and third fractions are less rich in phytocompounds. If one compare the So with Ts extracts, it can be easily seen that common sage extract (Fig.1-A) has a higher amount of polyphenols that can be recovered than Ts extract (Fig.1-B), prepared in the same conditions. Also, in the case of TS extract, the ethanol-water mixture is more efficient solvent than ethanol (Fig.1-B and C), the first solvent enhanced the yield of extract formation with almost 3%. Lee *et al.* reported yields ranging from 5.7 to 9.7 % for So and 12.6 % for Ts hydro-acetonic (30/70 v/v) extracts, which are lower than the overall extraction yield of our both hydro-alcoholic and ethanolic extracts [26].

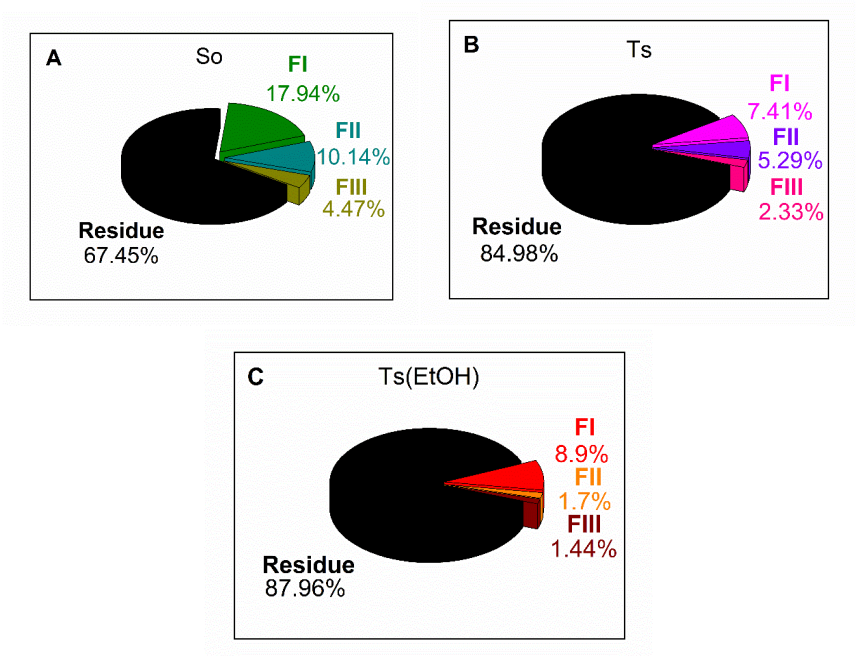


Fig. 1. Polyphenols amount in each extraction stage for **A**- So, **B**-Ts and **C**-Ts(EtOH) extracts

The total polyphenols content (TPC) was evaluated as gallic acid equivalents at both 765 nm (y=0.00945*x+0.017; R^2=0.9995) and 650 nm (y=0.00965*x+0.013; R^2=0.9998) wavelengths in the 50-450 μg/mL concentration domain, using Folin-Ciocalteu reagent and provided as an average value of four replicates (Fig. 2A). Based on the standard curve for gallic acid, the TPC was determined for each fraction as gallic acid equivalents (GAE) per g of extract. One can observe that in the case of So extract, TPC decreased slightly in each extraction stage being in the range of 194.13-217.44 mg GAE/g extract, while for Ts extracts the first fraction presents the highest TPC (239.65±5.11 mg GAE/g extract), while the second and the third fractions had a similar content of polyphenols (144.77-147.03 mg GAE/g extract). For Ts(EtOH) extract, surprisingly, the second fraction exhibited the highest amount of phenols (160.43±3.18 mg GAE/g extract), while the first and third fractions had a lower TPC, being between 78.68 and 100.45 mg GAE/g extract. Higher TPC values were obtained for So extract fractions than for Ts fractions. Also, the use of ethanol-water mixture enhanced the total polyphenols content in the case of Ts extract. The TP amount of our extracts are higher than that reported by Btissam *et al.* for the So hydro-alcoholic extract 30/70 v/v (162.23±0.36 mg GAE/ g extract) [8] or Lee *et al*. (40.5-96.2 mg GAE/ g extract and 118.7 ± 7.1 mg GAE/ g extract for So and Ts acetone-water extracts 70/30 v/v, respectively [26]. Ammamra *et al.*

reported a TP content of 97.11 ± 2.83 mg GAE/ g extract for Ts water extracts, which are lower than our TPC for Ts extract fractions [17].

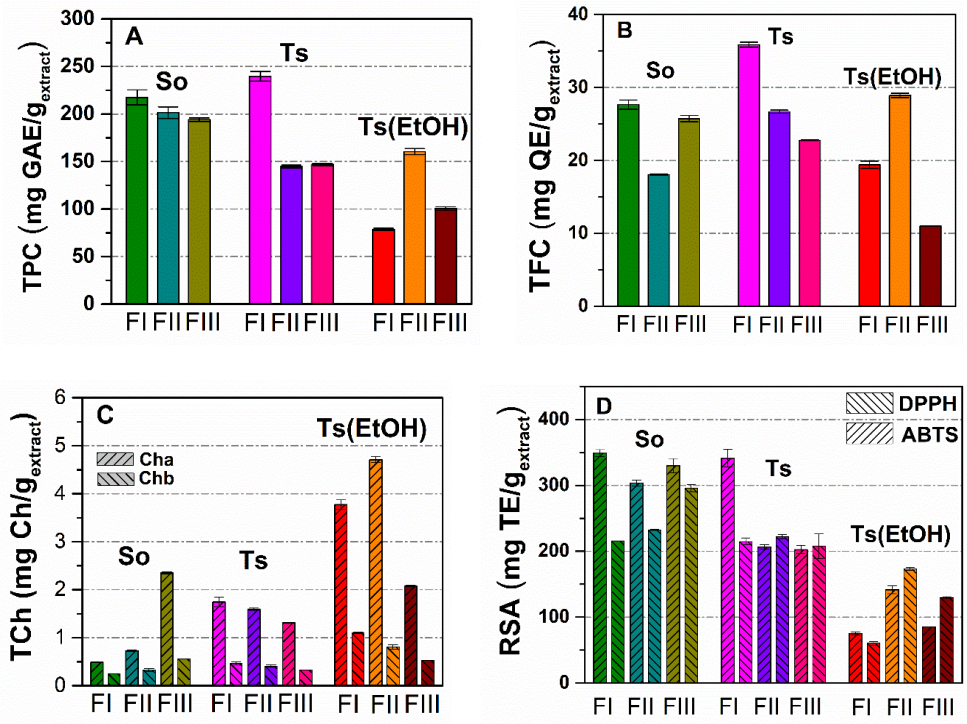


Fig. 2. Spectrophotometric determination of **A**- total polyphenols (as gallic acid equivalents, GAE), **B** - total flavonoids (as quercetin equivalents, QE), **C**- total chlorophyll pigment (TCh) and **D**- radical scavenger activity (RSA) as Trolox equivalents, TE

The total flavonoids content (TFC) was determined as quercetin equivalents (QE) in duplicate being assessed based on a standard curve established at 430 nm (y=0.04074*x, R^2=0.9991) using 2% aqueous solution of aluminium chloride in the concentration range of (5-50 µg/mL). For extracts with 1 mg/mL concentration, the solution absorbance was read at the maximum absorption wavelength, 420 nm, and the determined values are presented in Fig. 2C. The highest TFC for So extract was obtained in the first stage (27.66±0.63 mg QE/g extract), which was similar to that of the third fraction (25.74±0.43 mg QE/g extract) and higher than of the second fraction (18.08 ± 0.07 mg QE/g extract). In the case of Ts extract, a decrease of TFC was observed in each extraction stage with higher values than for So extract being in the range of 22.78-35.88 mg QE/g extract. For Ts(EtOH) extract, the second fraction was the richest in flavonoid compounds (28.93 mg QE/g extract), but a lower content than for Ts extract was observed. The TFC content of our Ts and Ts(EtOH) extract are mostly higher than that reported by Ammamra *et al.* for methanol, water, chloroform, ethyl acetate or

butanol Ts extracts, which were in the range of 3.20-21.92 mg QE/g extract [17]. Btissam *et al.* reported higher amount of TF (98.66 ± 0.65 mg/g extract) for So hydro-alcoholic extract (v/v=30/70) [8], but similar to that reported by Duletic-Lausevic *et al.* for ethanolic extracts (27.30 ± 8.48 mg/g extract) [2].

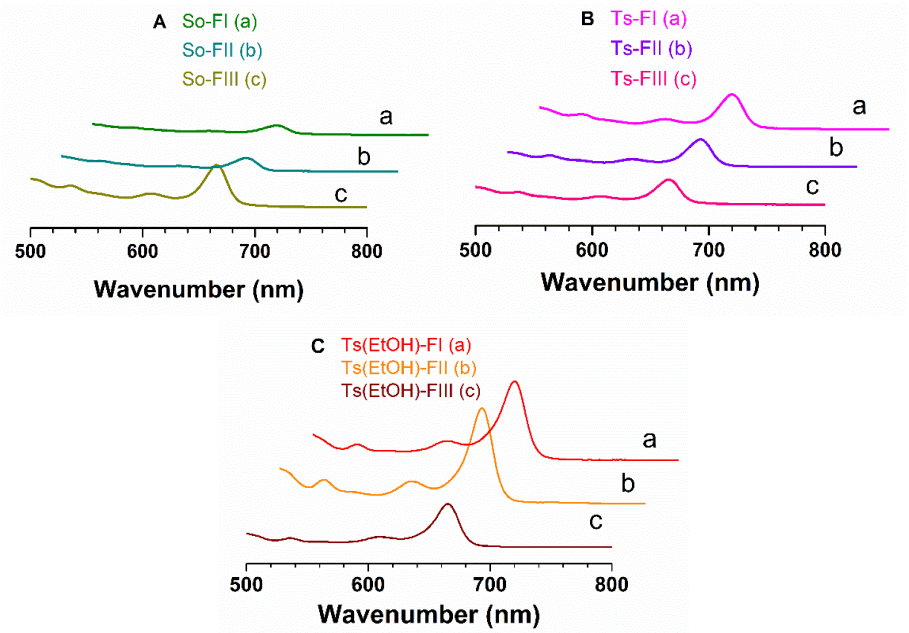


Fig. 3 UV-VIS spectra of extracts used for Ch determination: A-So, B-Ts, C-Ts(EtOH)

The chlorophyll pigments, Ch-a and Ch-b content values were determined by recording UV-Vis spectra for each sample at two different concentrations, and the solution absorbance values at 665 nm for Ch-a, 649 nm for Ch-b and 750 nm for removing the effect of solution colour, were introduced in the Ritchie's equations (eq. 1 and 2) [27].

$$Ch\text{-}a(\mu g) = 13.5275*(A_{665} - A_{750}) - 5.201*(A_{649} - A_{750})*DF*S \quad (1)$$

$$Ch\text{-}b(\mu g) = 22.4327*(A_{649} - A_{750}) - 7.0741*(A_{665} - A_{750})*DF*S \quad (2)$$

where *A* is the solution absorbance at a given wavelength, *DF*, dilution factor, and *S*, volume of solvent (ethanol or ethanol-water) in mL.

One can observe in Fig. 2C that Ch-a content is higher than that of Ch-b for all So and Ts extract fractions. In the case of So extract, the first two fractions had similar total chlorophyll content (0.75-1.06 mg TCh/g extract), while the third fraction had a higher amount (2.91±0.03 mg TCh/g extract) (Fig. 3A). Considering total chlorophyll content for Ts extract, the values are similar in all extraction stages (between 1.64 and 2.22 mg TCh/g extract), however a slight decrease of Ch amount could be observed (Fig. 3B). The highest recovery of Ch

was obtained for Ts(EtOH) extracts, especially in the first and second extraction stage (4.87-5.52 mg TCh/g extract) (Fig. 3C). The So extract fractions presented mostly higher amounts of total chlorophyll (0.93±0.05 mg TCh/g extract), a higher content only in the third fraction for Ts extract (1.33±0.06 mg TCh/g extract) and lower amounts for Ts(EtOH) extract (7.16±0.03 mg TCh/g extract) than global extracts obtained after three stage extraction that we previously reported [1].

Table 1.

Radical scavenging activity of polyphenolic extracts (DPPH assay)

Extract	IC50%	Correlation equation	R^2
So-FI	0.78	y=62.228*x+1.660	0.9951
So-FII	0.90	y=54.128*x+1.660	0.9954
So-FIII	0.82	y=58.838*x+1.660	0.9989
Ts-FI	0.80	y=60.808*x+1.660	0.9997
Ts-FII	1.31	y=36.797*x+1.660	0.9999
Ts-FII	1.34	y=36.089*x+1.660	0.9855
Ts(EtOH)-FI	3.60	y=13.432*x+1.660	0.9997
Ts(EtOH)-FII	1.91	y=25.256*x+1.660	0.9959
Ts(EtOH)-FIII	3.18	y=15.181*x+1.660	0.9855

The radical scavenging activity (RSA) of the extracts, determined by both DPPH and ABTS assays, using calibration curves for Trolox in 0.010-0.300 mg/mL concentration domain and plotting the RSA against the extract concentration (y=178.20*x+1.69, R^2=0.9983 for DPPH method and y = 79.298*x + 3.413 R^2=0.9984 for ABTS assay), are presented in Fig. 2D. Mostly, ABTS method led to obtaining lower values in comparison with DPPH assay, the first one being considered a better method to estimate antioxidant capacity [28]. RSA values of So fractions are higher than that for Ts and Ts(EtOH) fractions, being in the range of 303.46-348.99 mg TE/g extract (DPPH) and 215.55-295.34 mg TE/g extract (ABTS), the highest value being obtained for So-FI by DPPH assay and So-FIII through ABTS method. For Ts extract fractions, the RSA values are similar (202.39-220.03 mg TE/ g extract) for both assays apart from RSA value for Ts-FI (DPPH), which is higher (341.03±13.41 mg TE/g extract). For Ts(EtOH) fractions, the RSA values are significantly lower than for Ts extract, the highest values being obtained for Ts(EtOH)-FII, 141.64 and 173.23 mg TE/g extract for DPPH assay and ABTS method, respectively. Ben Farhat *et al.* reported antioxidant activity values for So methanolic extracts in the range of 159.03-392.48 mM TE/g extract that are lower than for all our So extract fractions (418.86-578.19 mM TE/ g extract) [29]. A lower antioxidant activity for So extracts was reported by Pasca *et al.* with 0.123±0.001 mmol TE/ mL in comparison with our extract fractions ranging 0.407-0.620 mmol TE/mL [5]. Also, Neagu *et al.* reported lower antioxidant activity of So hydroethanolic

extracts ranging from 544.89 to 627.82 µmol TE/g extract depending on the solvent/plant ratio, in comparison with our So extract fractions (861.2-1188.8 µmol TE/g extract), determined by ABTS assay [30]. From RSA of extract fractions were determined IC 50%, the concentration that inhibits 50% of DPPH free radicals from the solution (Table 1). One can observe that for extracts prepared using as extraction solvent ethanol-water mixture, the first fraction presents the lowest values of IC50%, except Ts(EtOH) extract for which the lowest value and consequently, the highest antioxidant activity were obtained for the second fraction, Ts(EtOH)-FII. HPLC-PDA analysis led to the identification of up to five compounds in extract fractions from the twenty-four available standard substances. Chlorogenic, caffeic and rosmarinic acids were identified in all extract fractions from both So and Ts plants. Additionally, caftaric acid was only identified in So extract fractions and protocatechuic acid was identified in both So and Ts extracts and in the first fraction of Ts(EtOH). The chromatograms are presented in Fig. 4 for So extract fractions, in Fig. 5 for Ts extract fractions and in Fig. 6 for Ts(EtOH) extract fractions, while the amount of polyphenolic compounds in each extract fraction was listed in Table 2.

Table 2.

Polyphenolic compounds identification and quantification by reverse phase HPLC-PDA for So and Ts extracts

	Standard substances				
	protocatechuic acid	caftaric acid	chlorogenic acid	caffeic acid	rosmarinic acid
RT (min)	6.982	10.920	13.376	15.378	32.353
Extracts	Concentration of standard substances in extract (mg/g extract)				
So-FI	0.188± 0.002	1.120± 0.014	1.270± 0.004	1.839± 0.012	60.746± 0.387
So-FII	0.175± 0.002	0.825± 0.010	0.955± 0.009	1.666± 0.002	54.635± 0.018
So-FIII	0.272± 0.002	0.682± 0.006	0.796±0.015	1.888±0.090	54.851± 0.095
Ts-FI	0.268± 0.004	nd	1.018± 0.008	1.533± 0.014	51.518± 0.088
Ts-FII	0.189± 0.002	nd	0.594± 0.005	1.029± 0.004	28.373± 0.004
Ts-FII	0.201± 0.006	nd	0.446± 0.021	0.941± 0.000	20.335± 0.010
Ts(EtOH)-FI	0.079± 0.000	nd	0.139± 0.003	0.450± 0.000	20.006± 0.020
Ts(EtOH)-FII	nd	nd	0.425± 0.007	0.814± 0.000	42.816± 0.040
Ts(EtOH)-FIII	nd	nd	0.260± 0.003	0.368± 0.002	21.876± 0.014

Notes: RT-retention time, nd-not detected

Generally, the first fraction had the highest amount of polyphenols and the content of phytochemicals decreases in each extraction stage. However, a higher content of protocatechuic acid, caffeic acid and rosmarinic acid was noticed in the third fraction.

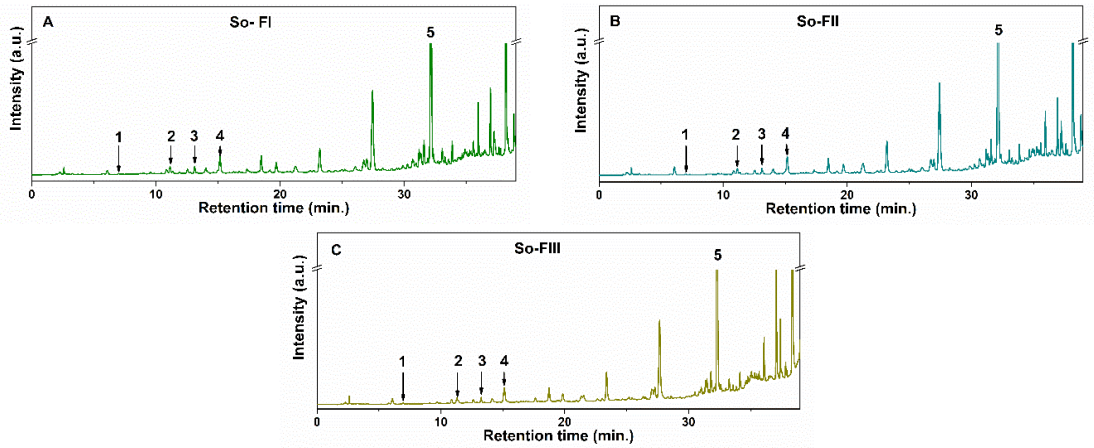


Fig. 4. HPLC-PDA chromatogramms for So extracts obtained in each stage at 279 nm (1-protocatechuic acid, 2-caftaric acid; 3- chlorogenic acid, 4- caffeic acid, 5- rosmarinic acid).

In comparison with So extracts, Ts extract fractions had lower content of each phenolic compound that were well correlated with their antioxidant activity and total polyphenols contents.

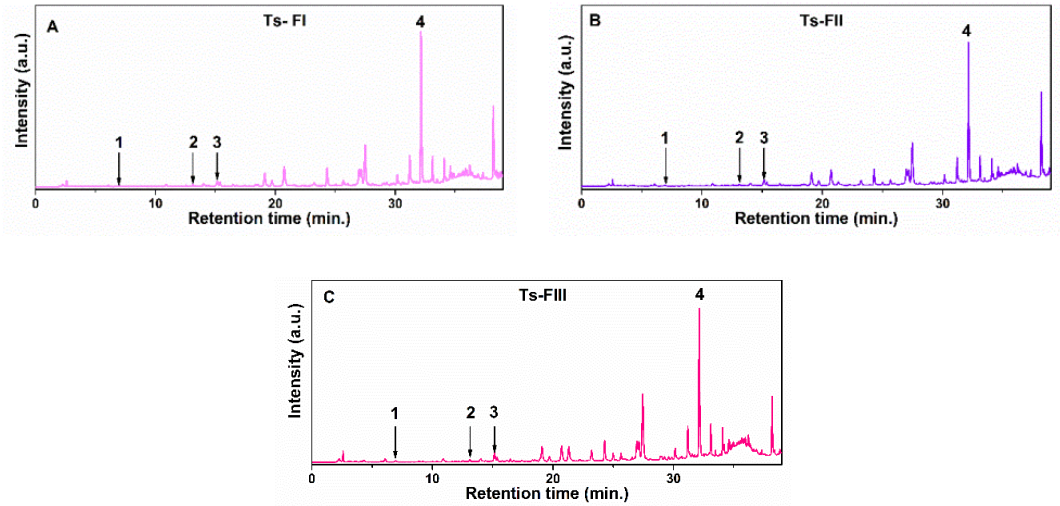
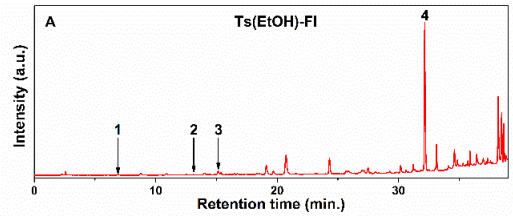
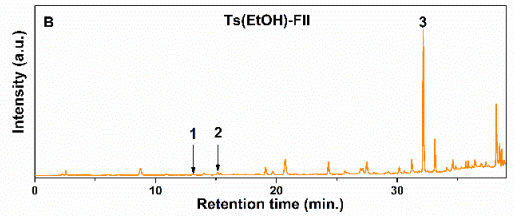


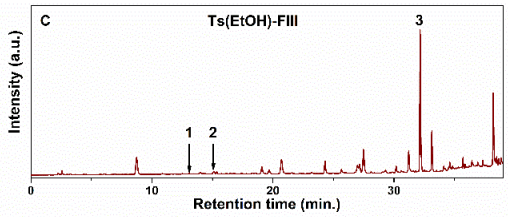
Fig. 5. HPLC-PDA chromatogramms for Ts- extracts obtained in each stage (1-protocatechuic acid, 2-chlorogenic acid, 3- caffeic acid, 4- rosmarinic acid).



1-protocatechuic acid, 2- chlorogenic acid, 3- caffeic acid, 4- rosmarinic acid.



1-chlorogenic acid, 2- caffeic acid, 3- rosmarinic acid.



1- chlorogenic acid, 2- caffeic acid, 3- rosmarinic acid.

Fig. 6 HPLC-PDA chromatogramms for Ts(Conv)-1 extracts obtained in each stage and overall extract at 279 nm.

However, in Ts extract fractions were identified large amounts of polyphenols as follows: protocatechuic acid (0.189-0.268 mg/g extract), chlorogenic acid, (0.446 -1.018 mg/g extract), caffeic acid (0.941-1.553 mg/g extract), and rosmarinic acid (20.006-51.518 mg/g extract).

The lowest amount of polyphenols was identified in Ts(EtOH) extract, for which three compounds were identified in all fractions. Protocatechuic acid was found only in the first fraction (0.079±0.000 mg/g extract) of Ts(EtOH). The highest amount of chlorogenic acid was noticed in the second fraction of Ts(EtOH) extract, which can be correlated with an enhanced radical scavenger activity and total polyphenols content. Thus, the phenolic compounds were chlorogenic acid (0.139-0.425 mg/g extract), caffeic acid (0.36-0.814 mg/g extract) and rosmarinic acid (20.003-42.816 mg/g extract).

Kozics *et al.* reported for So hydro-alcoholic extracts (60/40 v/v) contents of protocatechuic acid (0.01 mg/g extract), caffeic acid (0.68 mg/g extract) and rosmarinic acid (16.33 mg/g extract), which are all lower than the polyphenolic composition of our extract fractions [31]. An ethanolic extract of Ts prepared at room temperature for 24 h was reported to contain protocatechuic acid (1.03±0.18 mg/g extract), chlorogenic acid (2.24±0.40 mg/g extract), caffeic acid (1.27±0.196 mg/g extract) and rosmarinic acid (12.5±2.63 mg/g extract) having mostly higher amount of polyphenols than our Ts(EtOH) extract fractions, but a lower amount of rosmarinic acid [32].

4. Conclusions

The hydroethanolic (1/4 v/v) extract fractions from *Salvia officinalis* L. and hydroethanolic (1/4 v/v) and ethanolic extract fractions from *Thymus serpyllum* L. were prepared at 80°C by conventional method using the same vegetal material/solvent ratio. The aim of this study was to assess the phenolic compounds distribution during the extraction stages and their yields. Thus, besides the group spectrometric determination of total polyphenols, flavonoids, and chlorophyll pigments content of the extract from each extraction stage, the chemical profiling of each extract fraction was evaluated by reverse-phase HPLC-PDA analysis. In all fractions of extracts, rosmarinic acid was in the highest amount. Also, in all extract fractions from both common sage and wilde thyme were identified chlorogenic, caffeic acids, and protocatechuic acid, except the second and third fractions of Ts(EtOH), while caftaric acid was quantified only in the common sage extract fractions.

The utilization of ethanol-water mixture as extraction solvent determined an enhanced phytochemicals recovery in comparison with absolute ethanol in all extraction stages. All fractions of common sage extract exhibited higher antioxidant activity than wilde thyme extract fractions that can be well correlated with their amounts of phytochemicals.

One can notice that the extraction process does not proceed as expected, namely obtaining the largest amount of phytocompounds in the first extraction stage.

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