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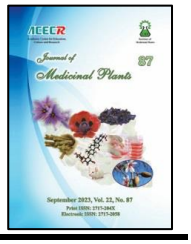

Research Article

Quantitative HPLC analysis of flavonoids and evaluation of antioxidant activity in two organs of *Salvia tebesana* Bunge

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ABSTRACT

Background: *Salvia tebesana* Bunge, as an important herb in folk medicine, is distributed in limited geographical locations in the Middle East. **Objective:** In this study, three protic solvents, 80 % methanol, 80 % ethanol, and double-distilled water, were investigated to recover the phenolic constituents from *S. tebesana* extracts. **Methods:** The antioxidant activity (estimated by total antioxidant capacity, DPPH, and FRAP radical scavenging assays) and the content of phenols, *ortho*-diphenols, phenolic acids, flavonoids, flavonols, and proanthocyanidins of extracts from leaves and stems of *S. tebesana* were investigated. After selecting the best solvent, the samples were assayed for individual flavonoid compounds (apigenin, quercetin, myricetin, and rutin) by HPLC-PDA. **Results:** Different plant extracts demonstrated strong radical scavenging activities, and the leaf extract obtained by 80% methanol showed the highest antioxidant capacity. The same extract also exhibited the most *ortho*-diphenol, phenolic acid, flavonoid, and proanthocyanidin contents (1598.5 ± 46.2, 742.2 ± 41.3, 487.6 ± 21.9, and 350.1 ± 31.4 mg 100 g^{-1} DW sample, respectively), while 80 % ethanol extract of leaves gave the most phenolic and flavonol contents (2299.2 ± 47.3 and 359.1 ± 42.3 mg 100 g^{-1} DW sample, respectively). The high level of flavone apigenin (466.1 ± 11.1 µg g^{-1} DW) and flavonol rutin (348.6 ± 10.02 µg g^{-1} DW) were also found in methanol extracts of leaves and are reporting for the first time in *S. tebesana*. **Conclusion:** These findings warrant that *S. tebesana*, in particular the leaves, can be suggested as a natural preservative in dietary and medical applications with the potential to reduce oxidative stress.

1. Introduction

The genus *Salvia* spp., with wide distribution all over the world, consists of nearly 900 species and, according to the Flora Iranica, is the largest genus of the *Lamiaceae* family. Different species of *Salvia* have various medicinal biological properties that make them used in folk medicine as remedies for the treatment of some disorders

Abbreviations: BHA, butylated hydroxyl anisole; BHT, butylated hydroxyl toluene; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EE, ethanol extract; FRAP, ferric reducing antioxidant power; HPLC-PDA, high-performance liquid chromatography with photodiode array detection; IC_{50}, the half maximal inhibitory concentration; ME, methanol extract; ROS, reactive oxygen species; TAC, total antioxidant capacity; TPTZ, 2,4,6-Tri(2-pyridyl)-s-triazine; WE, ultrapure water extract.

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such as seizure, ulcers, gout, rheumatism, inflammation, dizziness, headache, tremor, paralysis, diarrhea, hyperglycemia cerebral ischemia and memory disorders, as well as hepatitis [1]. It has been confirmed that the essential oil of *Salvia officinalis* L., *Salvia santolinifolia* L., *Salvia mirzayanii* L., *Salvia triloba* L., *Salvia repens* L., and *Salvia runcinata* L. show the best antibacterial activity [1, 2].

S. tebesana, with the local name of "Maryamgoli Tabasi" is a rare species, endemic in limited geographical locations in Afghanistan, Pakistan, and particularly in Iran [3]. In traditional Iranian medicine, the aerial parts of the plant are used for the treatment of treating colds, bronchitis, and tuberculosis, the relief of pain and the treatment of ulcers. Nevertheless, only a few works of literature exist on secondary metabolites from *S. tebesana*. These studies focused on some essential oil composition [4], four specific diterpenoids including tebesinin A, tebesinin B, aegyptinones A and B [3] and some main phenolic acids [1]. Studies have also shown antihyperglycemic, antihyperlipidemic, liver-protective (Aramjoo et al., 2022) and anti cancer activities (Asili et al., 2021), as well the antimicrobial (against both Gram-positive and Gram-negative strains), and cytotoxic properties (Eghbaliferiz et al., 2019) of the extract obtained from the root of *S. tebesana*.

Reactive oxygen species (ROS) are chemically reactive species naturally produced during cellular metabolism. Whenever the equilibrium between free radicals production and subsequent neutralization by antioxidant systems is disturbed, it leads to oxidative stress [8,9]. Although the use of synthetic antioxidants [butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), and α-tocopherol] in the food and pharmaceutical industry is a way to control ROS levels, due to their potential toxicity effects, there is a growing day by day in the natural substances exhibiting antioxidant activities.

Phenolic compounds possess the ability to quench the radicals depending upon the chemical structure, the molecular weight, and the arrangement of functional groups (hydroxyl or methoxy) about the structure [8]. Besides, the biosynthesis of these secondary metabolites is a dynamic process, mostly dependent on the environmental condition and the plant itself. Among the second group of factors, the most important one is the type of parts or organs in the plants which are used.

Extraction is the first important step for the isolation, quantification, and characterization of secondary metabolites from plant material. The solvent system (the type, degree of purity, and polarity) will surely play a key role in the extraction capacity of polyphenols and their antioxidant activities [10, 11]. However, the differences in the stability and solubility of phenolic compounds from plant materials extracted with varied solvents emphasize that the appropriate selection of extracting solvent is not as simple as it may seem. So this needs to be investigated, selecting the suitable solvent for the extraction recovery of polyphenols from each plant species. In this regard, the current study was aimed (i) to evaluate the efficiency of different solvent systems in extraction recovery of phenolic compounds from two organs of *S. tebesana* (ii) to determine the antioxidant properties and the content of phenolic subgroups obtained using the various solvents, and (iii) to analyze and quantify individual flavonoid contents by HPLC for the first time.

2. Materials and methods

2.1. Plant material

Aerial parts of the wild-growing plant of *S. tebesana* were collected from the 'Pikouh' and

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'Nissan' districts of Tabas, in South Khorasan province (Iran). The samples were then identified by comparison with authentic specimens deposited at the Herbarium of Research Center for Plant Sciences, Ferdowsi University of Mashhad (Voucher number 9640) (Fig. 1).

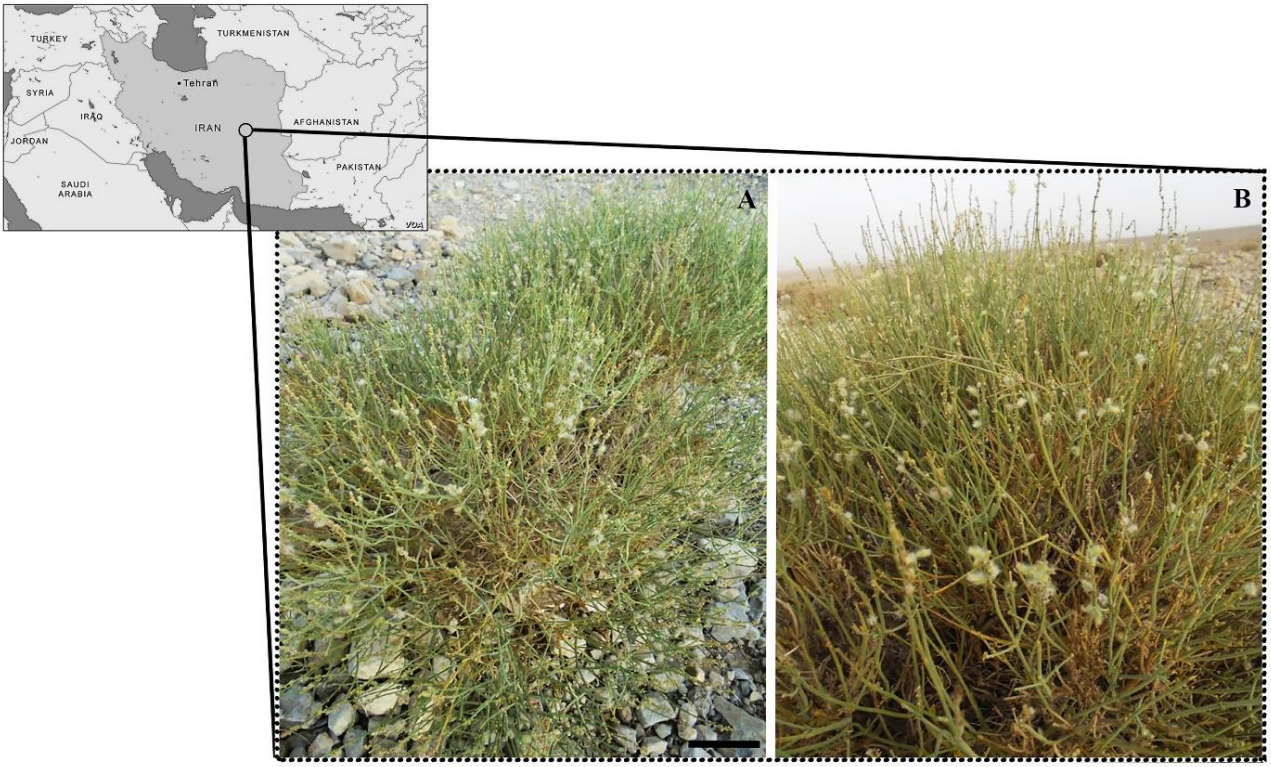

Fig. 1. The herb tested, *S. tebesana*; a complete plant in nature (A) and a view of its flowers (B). Bar=15 cm.

2.2. Sample preparation and extraction

The aerial parts of the plant were air-dried at 28 ± 2 ºC in a dark. Then, two parts of leaves and stems were separated and ground in a laboratory mill-mixer. The powdered samples (1 g) were individually mixed with 20 mL of double-distilled water. The extraction was continued in an ultrasonic bath at 48ºC for 20 min and then were centrifuged at 3000 rpm for 15 min, and evaporated under the fume hood. The same processes were followed for the preparation of sample extract in 80 % methanol (80 % MeOH, v:v) and 80 % ethanol (80 % EtOH, v:v).

The extract yields were calculated as Yield (%) = W_e/W_p ×100, Where W_e: the weight of 'dried extract' and W_p: the weight of the initial powdered plant.

To obtain the final 'sample extracts' for analysis, a 'dried extract' (1 mg) of each organ was extracted by 1 mL of the previous solvents (80 % MeOH, 80 % EeOH, and double-distilled water) and mentioned as methanol extract (ME), ethanol extract (EE) and ultrapure water extract (WE) and expressed as milligrams per milliliter.

2.3. Determination of total phenolic content

Total phenolic contents of the extracts were quantified by literature methods involving Folin–Ciocalteu's phenol reagent and gallic acid standard. Properly diluted extract of 200 µL was added to 2.5 mL of Folin–Ciocalteu's reagent

(10-fold diluted with distilled water). After 3 min, 2 mL of sodium carbonate anhydrous (7.5 %, w:v) was added and vigorously mixed on a vortex mixer. Following 30 min incubation in the dark, the absorbance was measured at 760 nm. Phenolic content of extracts was expressed as mg gallic acid equivalents per 100 g of dry weight [12].

2.4. Determination of ortho-diphenol content

An aliquot (100 µL) of each extract was mixed with 2 mL of aqueous methanol (50 %, v:v) and 0.5 mL of sodium molybdate solution (5 %, w:v) and shaken vigorously. After 15 min incubation at 25±2ºC, the absorbance (at λ_{max} 370 nm) was calculated from the calibration curve of caffeic acid and expressed as mg of CA per 100 g of dried sample [13].

2.5. Determination of phenolic acid content

The content of phenolic acid compounds was measured by Matkowski et al. [14] with slight modifications. The 0.5 mL of ME, EE, and WE of two organs were mixed with 2.5 mL of double-distilled water and 1 mL of HCl (5M). Afterward, 1 mL of Arnow's reagent was added to the mixture. After 5 min, 1 mL of NaOH (8.5 %, w:v) and 5 mL of double-distilled water were added to the mixture, shaken, and allowed to react for 30 min. Phenolic acid content was quantified using caffeic acid equivalent and expressed as mg caffeic acid per 100 g of dry weight.

2.6. Determination of total flavonoid content

To assess flavonoid contents in extracts, 500 µL of each extract was mixed with 1.5 mL of aqueous methanol (80 %, v:v). After 5 min, 100 µL of aqueous aluminum trichloride (10 %, w:v), 100 µL of potassium acetate (1M), and 2.8 mL of double-distilled water were added to the mixture. The samples were incubated at 25 ± 2ºC for 30 min. The absorbance was measured at 415 nm and the content of flavonoid was expressed in terms of quercetin equivalent [15].

2.7. Determination of flavonol content

Total flavonols were determined using the method proposed by Miliauskas et al. [16]. 1 mL of aluminum chloride (2 %, w:v) and 3 mL of aqueous sodium acetate (5 %, w:v) were added to 1 mL of ME, EE, and WE of stem and leaf. After incubation at 25±2ºC for 150 min, the absorbance of the reaction mixture was recorded at 445 nm. The content of total flavonol compounds was calculated by a standard curve of rutin solution (mg RU 100 g^{-1} DW sample).

2.8. Determination of proanthocyanidin content

Proanthocyanidin was measured using the modified vanillin assay described by Sun et al. [17]. To 250 µL of diluted ME, EE, and WE of samples (to 1 mL) were added 1 mL of vanillin reagent (1%, w:v) and mixed thoroughly with a vortex mixer. Afterward, 2.5 mL of sulfuric acid (9N) was added to each tube and heated in a water bath at 38 ºC for 15 min. The absorbance was determined at 500 nm against blank for each solution and proanthocyanidin content of extracts was expressed in terms of catechin equivalent.

2.9. Total Antioxidant Capacity (TAC) assay

The antioxidant capacity has been evaluated based on Prieto et al. [18]. Briefly, 2.7 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added to 300 µL of each sample extract. Then, the reaction mixture was incubated at 95 ºC for 90 min. After recording the absorbance (695 nm) against the reagent blank, the TAC capacity of the samples and the ascorbic acid (in a concentration-dependent manner) were determined by using the following formula:

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TAC capacity (%) = [(control absorbance−sample absorbance)/(control absorbance)] × 100, and expressed as IC_{50} value (µg mL^{-1}).

2.10. Ferric Reducing Antioxidant Power (FRAP) assay

Different extracts (100 µL) from each organ were mixed with FRAP solution (3 mL), composed of 20 mM ferric chloride hexahydrate, 300 mM acetate buffer, and TPTZ (10 mM) in a ratio of 1:10:1 (v:v:v), respectively. Then absorbances were taken at 4 min after the start of the reaction at 593 nm and compared to the standard graph. Iron (II) sulfate was used as the standard and analyzed as above [19].

2.11. DPPH radical scavenging assay

Briefly, 50 µL of extract solutions (ME, EE, and WE) was dissolved in 2.5 mL of methanol containing DPPH (0.00004 %, w:v). After 30 min of incubation in the dark, absorbance was measured at 517 nm. The negative control was set using methanol with DPPH (1 mL). The results were calculated as the percentage of DPPH absorption inhibition as follows [19]:

Scavenging effect (%) = $[(A_{DPPH}–A_S)/A_{DPPH}] \times 100$

Here, A_S: mixture absorbance with sample; A_{DPPH}: Mixture absorbance of the DPPH solution (without sample). IC_{50} value (µg mL^{-1}) was the extract concentration required to cause a 50 % decrease of the absorbance at 517 nm and calculated from the graph plotting extract concentration against inhibition percentage.

2.12. Analysis of individual flavonoid compounds by HPLC-PDA

Sample preparation. The samples were prepared according to Fernández-Lorenzo et al. [20] with some modifications. Dried samples of both organs were ground to a fine powder, and 0.1 g quantities were extracted at 25 ºC with 3 mL of HPLC grade 80 % methanol. The samples were then centrifuged at 3000 rpm for 30 min. The supernatants were evaporated to dryness under the fume hood, and then the residue was dissolved in 2 mL HPLC grade methanol, centrifuged at 3000 rpm for 30 min, and transferred to a vial.

For analysis of flavone and flavonols in the extract, 5 mg of the extract was dissolved in 2 mL of distilled water and then hydrolyzed in a solution of 0.5 mL 6M hydrochloric acid and 2.4 mL HPLC grade ethanol. After refluxing at 90 ºC for 120 min, the extract was made up to 5 mL, and sonicated [21]. Each extract solution was filtered using a 0.45-µm filter (Minisart) before injection.

HPLC analysis. Flavonoid compounds were analyzed using an HPLC-PDA (Agilent1260) with a photodiode array detector (SPD). A C-18 (Phenomenex) reverse phase column (4.6 × 100 mm, 3.5 µm particle size) with a temperature of 25 ºC was used. The mobile phase consisted of double-distilled water with 0.2 % glacial acetic acid, pH 3 (solvent A) and HPLC grade methanol (solvent B). A linear gradient of solvent B was applied from 5 % to 100 % for 30 min, followed by an isocratic washout phase of 100 % solvent B for 10 min before returning to the initial conditions. The injection volume of a sample was 5 µL, and the constant solvent flow rate was 1 mL per min. The chromatograms were monitored at 365 nm. The identification of chemical compounds was based comparing UV spectra and retention times of authentic commercially available reference substances (apigenin, quercetin, myricetin, and rutin; purchased from Sigma–Aldrich) with the compounds presented in analyzed extracts. The stock solution of each standard (1 mg mL^{-1}) was diluted to five different concentrations (4, 8, 16, 32, 64 µg mL^{-1}) that

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were fed individually to the HPLC system and the calibration curve obtained. The flavonoid compounds in the 80 % methanolic extract of each organ were expressed in micrograms per gram of dry weight (DW). The validation data of quantitative analysis of individual flavonoids by HPLC-PDA analysis is shown in Table 1.

Table 1. Retention time, Relative standard deviation, Regression coefficient, Limit of detection, and Limit of quantification of standards for HPLC method validation.

Name of Standard	Detected at wavelength (nm)	Retention time	RSD (%) of retention time	Regression Coefficient R^2	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
Rutin	365	21.035	0.071	0.991	0.080	0.243
Myrcetin	365	25.987	0.050	0.991	0.089	0.270
Quercetin	365	27.711	0.053	0.994	0.094	0.285
Apigenin	365	31.467	0.082	0.993	0.073	0.222

Note: LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation.

Statistical analysis. One-way analysis of variance (ANOVA) and regression was performed using SPSS Statistics Version 16, and Duncan's multiple range test was used to determine the significance of differences among their mean values ($P < 0.05$). The t-test analysis was also done at a 95 % confidence interval and $P < 0.05$ for HPLC data. All experiments (sample extraction and biochemical assays) were carried out in triplicate and expressed as mean ± standard deviation.

3. Results

In the first part of this study, solvent-type effects on the extraction capacity from two organs of *S. tebesana*, leaves and stems, were assessed and shown in Table 2. Extraction yields ranged from 0.6 % to 5.6 % in WE of stem and ME of leaves, respectively. In both leaves and stem, the highest biomass was related to aqueous methanol, so the extraction yield in each organ followed the order: ME>EE>WE. Besides variation in extraction yield depending on the solvent type, it was noteworthy that all the extraction extent in leaves of *S. tebesana* were greater than the stem yields.

Table 2. Extraction yield of *S. tebesana* organs, obtained by distilled water, 80% ethanol and 80% methanol

	Extract Yield (%)	
Extract Sample	**Stem**	**Leaf**
Water Extract	0.6 ± 0.001^{c}	4 ± 0.007^{c}
Ethanol Extract	1.6 ± 0.002^{b}	4.4 ± 0.009^{b}
Methanol Extract	2.8 ± 0.002^{a}	5.6 ± 0.011^{a}

Note: Values are mean ± standard deviation, n = 3. For each solvent, values in the same column bearing different letters are significantly different at $P < 0.05$ (at least).

The current study was also designed to evaluate the influence of extraction by protic solvents (with different polarity) on phenolic derivatives content of two organs. Regardless of the solvent type employed for extraction, the leave of *S. tebesana* was significantly the richer source of phenolics, *ortho*-diphenols, phenolic acids, and flavonoids than its stem. According to the obtained results, the amount of total phenolics in leaves and stems varied in different extracts ranging from 67.5 to 2299.2 mg 100 g^{-1} dry sample (Table 3). Although 80 % EtOH was better than two other solvents in extraction of leaf phenolic compounds, 80 % MeOH efficiency for extraction of total phenolics of the stem was the highest.

The content of *ortho*-diphenols from *S. tebesana* extracts differed significantly depending on the plant organ and the solvent used for extraction. Based on obtained results, the

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richest in *ortho*-diphenols were methanol extracts of both leaves and stem (1598.5 and 632.7 mg 100 g^{-1} dry sample, respectively), and the lowest ones were in water extracts of analyzed organs (534.5 and 128.1 mg 100 g^{-1} dry sample for leaves and stem, respectively) (Table 3).

Table 3. Total phenolics, *ortho*-diphenols, phenolic acids, and flavonoid derivatives content of different organs from *S. tebesana*, extracted with distilled water, 80 % ethanol, and 80 % methanol.

Constitute	Phenolic cont. mg GA 100 g^{-1} DW		*ortho*-di phenol cont. mg CA 100 g^{-1} DW		Phenolic acid cont. mg CA 100 g^{-1} DW	
Extract Sample	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**
Water Extract	67.5±1.1^{f}	618.1±15.3^{de}	128.1±9.4^{d}	534.5±24.2^{c}	26.6±0.9^{f}	60.3±1.1^{e}
Ethanol Extract	610.2± 17.2^{de}	2299.2±47.3^{a}	466.4±13.2^{c}	1231.3±54.1^{b}	117.8±12.3^{d}	537.1±35.5^{b}
Methanol Extract	725.7±22.1^{c}	1923.1±35.1^{b}	632.7±23.2^{c}	1598.5±46.2^{a}	205.2±11.9^{c}	742.2±41.3^{a}

Note: Values are mean ± standard deviation, n = 3. For each solvent, values in the same column bearing different letters are significantly different at P < 0.05 (at least).

Table 3. Total phenolics, *ortho*-diphenols, phenolic acids, and flavonoid derivatives content of different organs from *S. tebesana*, extracted with distilled water, 80 % ethanol, and 80 % methanol (Countinued)

Constitute	Flavonoid cont. mg QE 100 g^{-1} DW		Flavonol cont. mg RU 100 g^{-1} DW		Proanthocyanidin cont. mg CAT 100 g^{-1} DW	
Extract Sample	**Stem**	**Leaf**	**Stem**	**Leaf**	**Stem**	**Leaf**
Water Extract	27.9±0.8^{e}	116.1±22.5^{d}	13.7±0.7^{c}	107.2±11.1^{c}	34.4±0.6^{d}	260.6±17.3^{b}
Ethanol Extract	322.0±18.7^{c}	361.8±17.8^{b}	111.4±20.2^{c}	359.1±42.3^{a}	80.7±1.9^{d}	281.0±25.6^{b}
Methanol Extract	356.2±16.4^{b}	487.6±21.9^{a}	139.5±19.1^{c}	333.2±38.2^{b}	181.1±8.9^{c}	350.1±31.4^{a}

Note: Values are mean ± standard deviation, n = 3. For each solvent, values in the same column bearing different letters are significantly different at P < 0.05 (at least).

Assessment of phenolic acid content of *S. tebesana* revealed the maximum accumulation in the leaves extracted by 80 % MeOH (742.2 mg 100 g^{-1} dry sample). The contents of these compounds in individual anatomic organs were in followed order: ME>EE>WE (the leaf extracts higher than in the extracts of stem).

Except for flavonol content, extraction into 80 % MeOH gave the highest amount of flavonoids and proanthocyanidins, whereas water afforded the lowest amounts (Table 3). Although methanol and ethanol extracts of stem were moderately abundant in flavonoid compounds (356.2 and 322.0 mg 100 g^{-1} dry sample, respectively), the methanol extract of leaves was the richest in flavonoids (487.6 mg 100 g^{-1} dry sample).

The antioxidant potential of different extracts was screened by three complementary test systems, namely TAC, DPPH radical scavenging, and FRAP radical scavenging assays. As can be seen in Fig. 2A, the IC_{50} values of TAC for methanol extract of leaves was 39.01 ± 0.3 µg $mL^{-1}$and that was statistically (P < 0.05) similar to the total antioxidant capacity of ascorbic acid (37.87 ± 1.1 µg mL^{-1}). This shows the highest antioxidant capacity for methanol extract of leaves followed by ethanol extract of the same organ. According to TAC, all the extracts of stem were less active than the corresponding extracts of leaves. Based on Fig. 2B, the extraction sample, obtained with 80 % MeOH from leaves and stem, gave the highest FRAP radical scavenging activities (1944.3 and 917.3 µg Fe II 100 g^{-1} DW, respectively), while the other extracts show the lowest activities. As shown in Fig. 2C, when the mean values of IC_{50} for the different extraction solvents of DPPH radical scavenging activity were compared, the experimental data were observed to be in the range of 47.7 µg mL^{-1} to 251.1 µg mL^{-1}. In contrast, the IC_{50} value of ascorbic acid was 3.4

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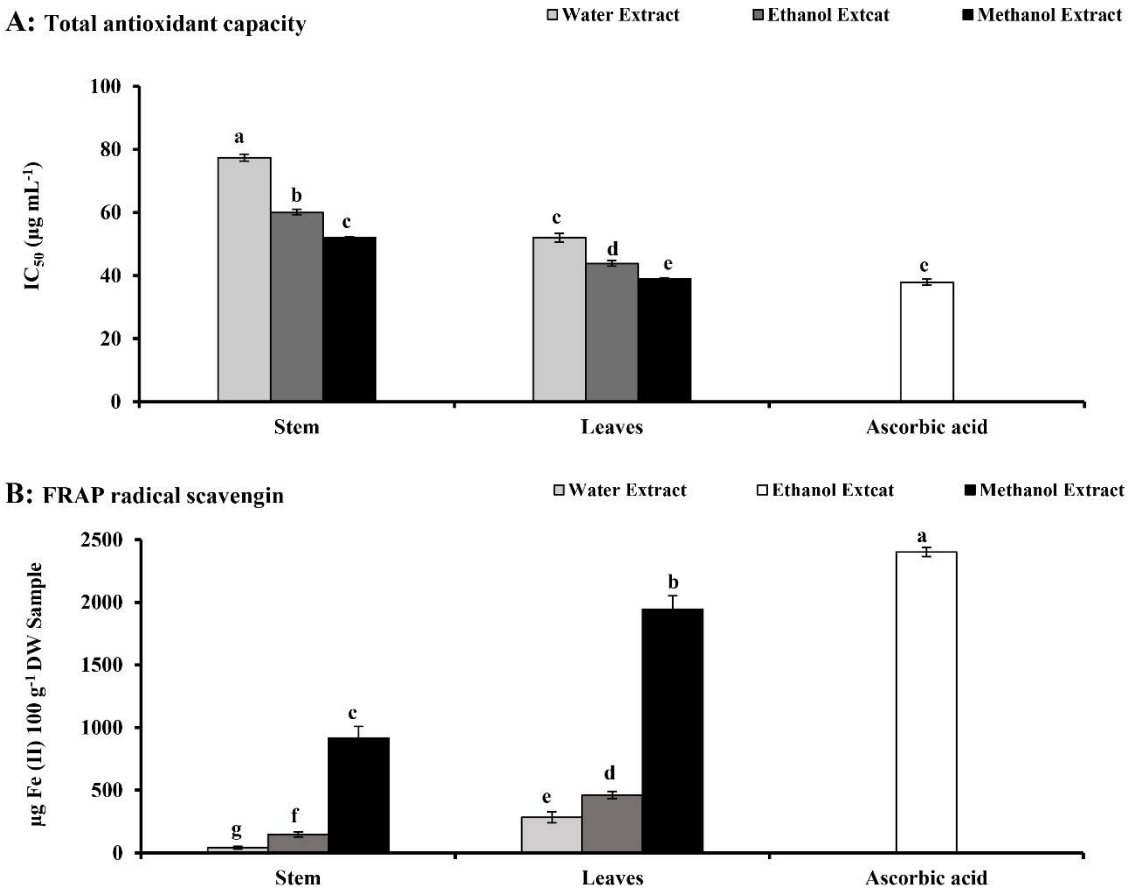
µg mL^{-1}. Maximum scavenging activity in the samples studied was observed in ME and EE of leaves with the lowest IC_{50} values. While there were moderate statistically significant correlations between total phenolic compound of extracts and TAC and DPPH scavenging assays ($r^2 = 0.798$ and $r^2 = -0.764$, $P < 0.05$ respectively), correlation with FRAP assay was somewhat weaker ($r^2 = 0.577$) (Table 4). Whereas the method of TAC and FRAP scavenging assays were strongly correlated with the contents of *ortho*-diphenol, phenolic acid, and proanthocyanidin; the DPPH antioxidant assay had a strong correlation ($r^2 = -0.840$) only with flavonoid compounds which can be attributed to their high contents (Fig. 3A-C).

The methanol extract from two organs of *S. tebesana* differed significantly for the content of four flavonoid compounds: the flavone apigenin and the flavonols quercetin, myricetin, and rutin. Rutin and myricetin appeared first at the retention time values of 21.035 and 25.987, respectively, then quercetin and apigenin at 27.711 and 31.467, respectively (Fig. 4A). A significant component, apigenin, followed by rutin as another prevalent compound, were indicated the highest amount in leaves (466.1 ± 11.1 and 348.6 ± 10.02 µg g^{-1} DW, respectively) compared to stems (322.2 ± 39.98 and 232.3 ± 10.67 µg g^{-1} DW, respectively) (Fig. 4B). Although the content of quercetin in both studied organs was to some extent similar, the compound myricetin was not detected in the stem extract.



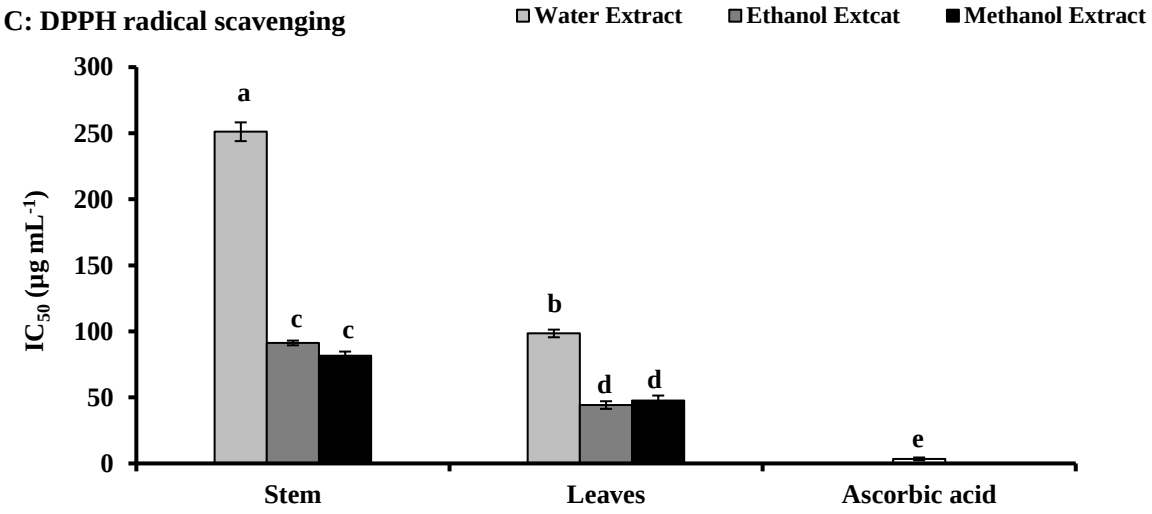


Fig. 2. Total antioxidant capacity (A), FRAP radical scavenging (B), and DPPH radical scavenging (IC_{50}) (C) of different extracts from *S. tebesana*. The values are reported as mean ± standard deviation, n = 3.

Table 4. A correlation matrix with the Pearson coefficient values for the phenolic derivatives contents and antioxidant activities of different organs from *S. tebesana.*

Parameters	Phenolic cont.	*Ortho*-diphenol cont.	Phenolic acid cont.	Flavonoid cont.
Phenolic cont.	1			
***Ortho*-diphenol cont.**	0.936	1		
Phenolic acid cont.	0.920	0.980	1	
Flavonoid cont.	0.748	0.828	0.816	1
Flavonol cont.	0.996	0.961	0.943	0.897
Proanthocyanidin cont.	0.805	0.880	0.784	0.601
TAC assay	0.798	0.956	0.923	0.784
DPPH assay	-0.764	-0.775	-0.664	-0.854
FRAP assay	0.577	0.815	0.816	0.761

Table 4. A correlation matrix with the Pearson coefficient values for the phenolic derivatives contents and antioxidant activities of different organs from *S. tebesana* (Countinued)

Parameters	Flavonol cont.	Proanthocyanidin cont.	TAC assay	DPPH assay	FRAP assay
Phenolic cont.					
***Ortho*-diphenol cont.**					
Phenolic acid cont.					
Flavonoid cont.					
Flavonol cont.	1				
Proanthocyanidin cont.	0.826	1			
TAC assay	0.843	0.900	1		
DPPH assay	-0.793	-0.774	-0.716	1	
FRAP assay	0.644	0.777	0.930	-0.550	1

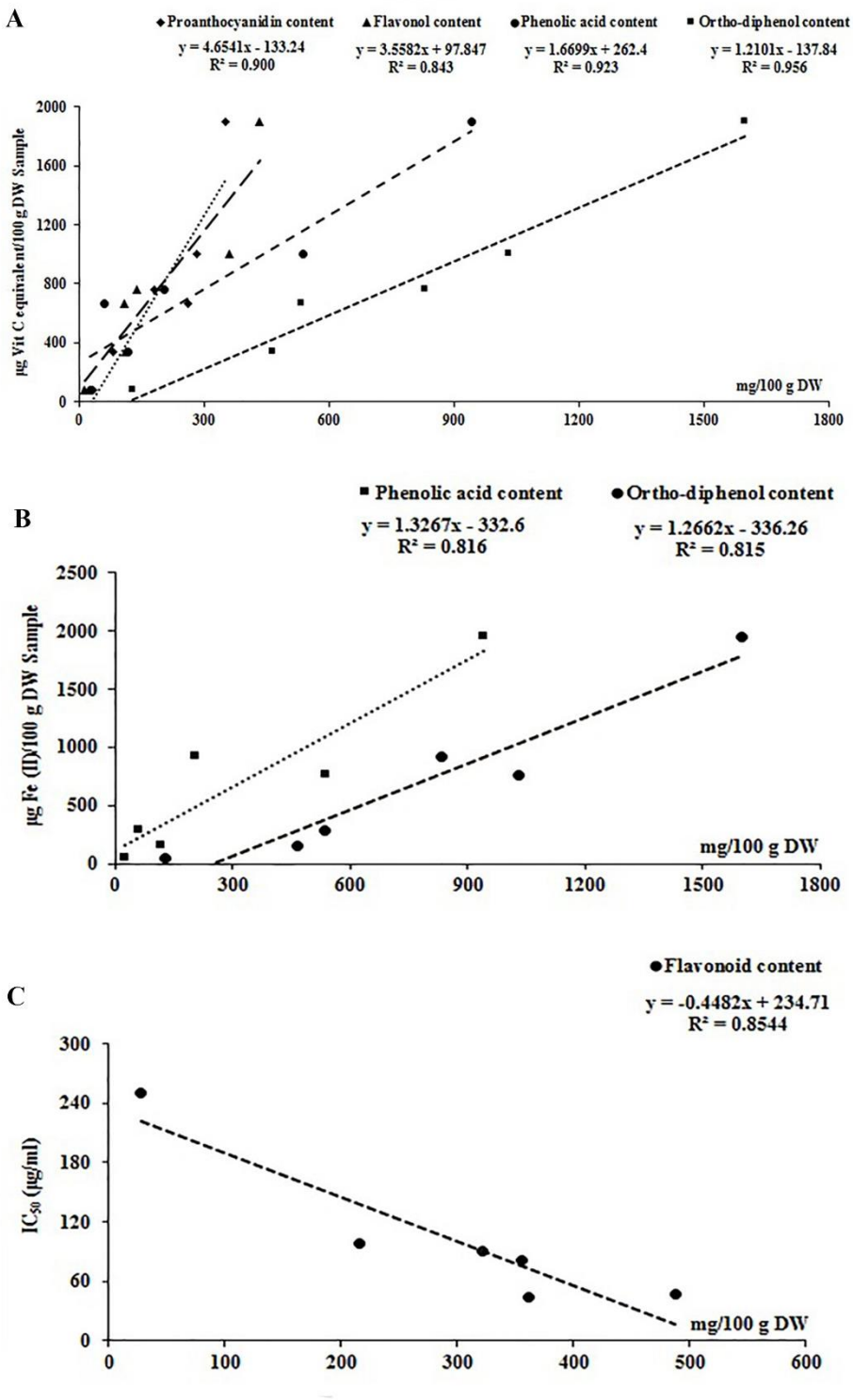


Fig. 3. Linear regression analysis for demonstration of the correlation coefficient between phenolic derivatives and total antioxidant capacity (A), FRAP radical scavenging (B), and DPPH radical scavenging (C) with the strongest relationships.

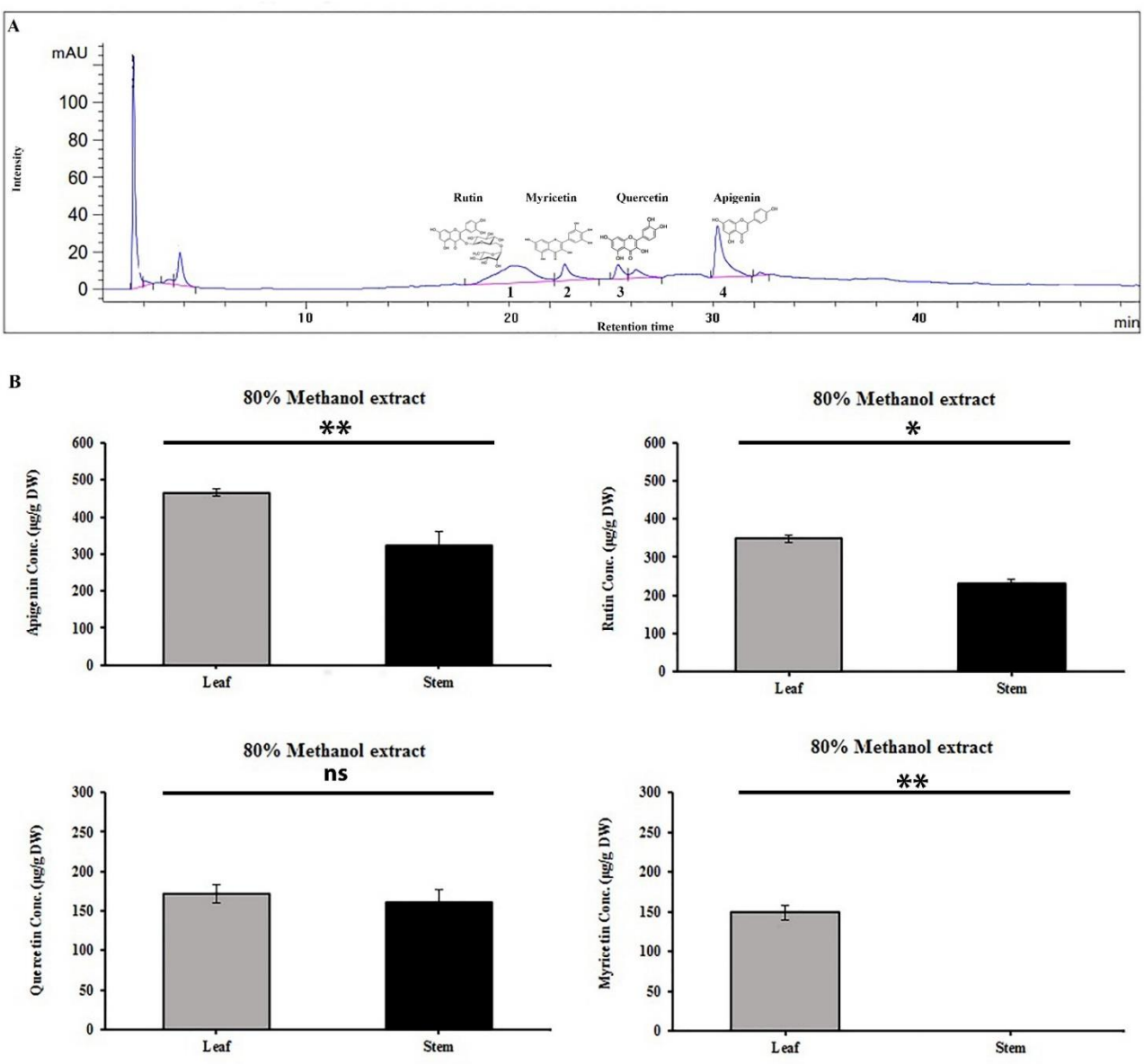


Fig. 4. Comparison of individual flavonoid content in leaf and stem of *S. tebesana*. A Chromatogram of all standard mixture recorded at 365 nm - Rutin (1), Myricetin (2), Quercetin (3), and Apigenin (4)(A), and individual flavonoids contents (µg g^{-1} dry weight ± standard deviation)(B). Bars display the mean of triplicate results (mean ± standard deviation). The statistical test performed is a t-test ($P < 0.05$). ns: not significant, *: significant ($P < 0.05$), **: very significant ($P < 0.01$).

4. Discussion

It has been confirmed that obtaining high biomass yields, and consequently the most desirable secondary metabolites, depends on the appropriate selection of extraction solvent for each plant species. The highest extract yields of aqueous methanol in our findings are in agreement with the previous investigations reported that maximum biomass was obtained with aqueous methanol [22]. Studies on aerial

parts of *Stachys turcomanica* Trautv. showed that 80 % methanol could be a better solvent for extracting the target compounds with the highest yield biomass in comparison with water and other aqueous methanol solvents [11]. Hayouni et al. [10] reported that aqueous solvent mixtures (acetone/water/HCl or ethyl acetate/methanol/water) were superior to single solvents (acetone or chloroform) for extracting with more yield percentage from *Tunisian Quercus coccifera* L. and *Juniperus phoenicea* L. fruits. However, studies by Barchan et al. [23] on three *Mentha* species showed that the highest yields corresponded to methanol and water solvents, and the weakest yields corresponded to dichloromethane and n-hexane solvents. Many pieces of the literature confirmed that depending on the nature of extractable constituents, the high polarity of various solvents can cause effective differences in extraction efficiencies [9]. Although the polarity index of water (P W 10.2) is more than methanol and ethanol (P MeOH and EtOH 5.1 and 4.3, respectively) [24], an aqueous-alcoholic mixture (80 % MeOH and 80 % EtOH) facilitated more chemicals extraction of *S. tebesana* that are probably solvable in organic solvents and/or water, and therefore, the extraction efficiency with combined use of organic solvents and water could be higher than by using them alone.

The higher extraction extent in leaves than stem of *S. tebesana* was in line with the result of Fiore et al. [25] for six *Salvia* species. Nevertheless, the larger yields of extraction were obtained from the flowers and the whole plant of *Salvia amplexicaulis* Lam. in comparison with leaves and stem [26]. Končić et al. [27] showed that under the extraction with ethanol 96 %, the leaves of *Berberis* species had the most extraction yield among other organs, such as roots and twigs.

The dynamics of phenolic formation in various parts of a plant are associated with the function of these compounds in plants' lifecycle and the growth phase. For analyzed organs, the maximum extraction efficiency of phenolics was significant in hydroalcoholic solvents. On par with earlier observations of Wijekoon et al. [28], the lowest total phenols in our research were also obtained for WE. In an experiment, the total phenol content of leaves and stem for *Salvia amplexicaulis* were 222.40 and 173.67 (mg g^{-1} dry extract) for ethanol extracts, and 154.33 and 124.31 (mg g^{-1} dry extract) for methanol extracts [26]. Alimpić et al. [29] also refer that the variation of phenolic substations of *Salvia jurisicii* Kosanin depending on the plant part and solvent ratio. It was found that in comparison with pure solvents, 50 % methanol gave the best extraction ratio of phenolics for *Acacia polyacantha* Willd. gum, followed by ethanol 50 % extract [30]. The extraction efficiency of different solvents depends on both the solubility degree of plant materials as well as the solvent properties used for extraction [9]. The first factor is affected by the polarity of extractable constituents, the stereochemistry of the compounds (the polar and non-polar fragments of the molecule), the degree of polymerization, and the intermolecular interaction between them and the solvent or with other plant constituents [9, 23, 31]. While the second factor is influenced by the solvent structure (organic and inorganic ones) as well as the polarity, vapor pressure, viscosity, and dielectric constant of each solvent [9,28,31]. Despite the multifaceted nature of this issue, it has been opined that the higher polarity of solvents makes the better solubility for phenolic compounds [23]. By the way, in comparison with water, the lower polarity of methanol or ethanol favors the solubility and diffusion of phenolic

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compounds by reducing the dielectric constant of the solvent [31]. Another reason for the success of hydroalcoholic mixtures as solvents is avoiding the dehydration and collapse of plant cells, which makes the extraction of phenolic compounds easy [31].

In our study, *ortho*-diphenols in *S. tebesana* leaves and stem were found to be markedly higher in 80 % MeOH as compared to other solvents. The results obtained by Monteiro et al. [32] show that sample material and solvent type can severely influence the levels of extractable *ortho*-diphenols. Accordingly, for *Cucumis melo* L., the effective solvent employed for *ortho*-diphenols extraction was ethanol 96 % [33], while for Olive oils, 60 % methanol was used instead of the remaining solvents [34].

In the absence of adequate literature regarding phenolic acid quantity of different polarities extracts, Haminiuk et al. [35] studied the extraction capacity of phenolic acids from *Eugenia pyriformis* Cambess. using different solvents and stated that absolute and aqueous methanol (1:1 ratio) was the most effective extraction agents for the recovery of phenolic acids. In the present study, ME of both organs represented the highest phenolic acid content.

In the present study, flavonoid, flavonol, and proanthocyanidin contents significantly declined from alcoholics solvents to water, irrespective of organ type. Alimpić et al. [26] have made a comparative analysis of flavonoid content of *Salvia amplexicaulis* using different solvents, and concluded that although methanol extract of the whole plant (38.15 mg g^{-1} dry extract) showed higher content of flavonoids than its ethanol extract (27.35 mg g^{-1} dry extract), methanol extracts of other studied parts contained fewer flavonoids than the ethanol extracts [36]. For flavonoid extraction in three *Salvia* species, methanol and ethyl acetate were the most effective solvents in comparison with n-hexane and water [37]. In line with our results, Karatoprak et al. [38] and Haminiuk et al. [35] stated that aqueous methanol extract was a better extraction agent than other solvents in getting flavonols from *Salvia virgata* Jacq. and *Eugenia pyriformis*, respectively. By using different extraction solvents in another study, the flavonol content of *Acacia ataxacantha* DC. was in the order of ethyl acetate > methanol > hydroalcoholic > dichloromethane > n-hexane [39]. The maximum recovery of alcoholic or hydroalcoholic solvents for proanthocyanidins extraction has also been confirmed in many studies [28, 40]. It has been stated that 'low-viscosity' solvents have low density and high diffusivity, which allows them to readily diffuse into the pores of the plant matrices to leach out the bioactive constituents [28]. Based on the viscosity index of solvents in the present study (η value 0.89, 0.54, and 1.07 for water, ethanol and methanol, respectively) [41], methanol has the best ability to penetrate and subsequently extract metabolites.

In the present study, the DPPH antioxidant assay had a strong correlation only with flavonoid compounds. However, the methods of TAC and FRAP scavenging assays were strongly correlated with the contents of *ortho*-diphenol, phenolic acid, and proanthocyanidin [42]. The significant positive influence of phenolic derivatives contents on antioxidant activities was in accordance with the results of experiments on *Salvia nemorosa* [43], *S. tebesana* [44], *S. multicaulis* [45], *S. officinalis* [46], *S. verticillata* [47], and *S. miltiorrhiza* [48].

The obtained profile determined using HPLC-PDA clearly shows that leaves of *S. tebesana* contain more significant amounts of specific flavonoids than the stem. Flavonols are characterized by the presence of a hydroxyl

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group at C3 as compared to flavones that may exhibit structural variations of O-methylation, C-methylation, hydroxylation, and prenylation. The values of apigenin and rutin, which showed the highest content in methanol extract, are also higher than those reported by Kivrak et al. [37] measured in three species of *Salvia* (*S. potentillifolia, S. nydeggeri,* and *S. albimaulata*). HPLC chromatogram confirmed the presence of quercetin in both organs, while myricetin was not present in stem samples. Although the presence of quercetin [46] and myricetin [49, 50] was earlier reported for some species of *Salvia*, Kivrak et al. [37] could not detect quercetin and myricetin in the studied *Salvia* species. These results indicate that flavonoid content of *Salvia* species may change depending on the plant species.

5. Conclusion

Taking into account the results of this research, it was concluded that the percent recovery of antioxidant compounds of each organ of *S. tebesana* depends mainly on the polarity degree of the solvent being used, and polyphenols derivatives are most soluble in organic solvents (80 % methanol and 80 % ethanol) less polar than water. Content of individual flavonoids varied in a wide range (466.1-149.1 µg g^{-1} DW of sample), and apigenin and rutin were the most abundant flavonoid constituents. These results indicate that *S. tebesana*, in particular its leaves extracted by 80 % methanol, can be used as a natural preservative in dietary and medical applications with the potential to reduce oxidative stress.

Author contributions

M.B. carried out the laboratory work and composed the manuscript; M.CH and A.G. supervised the project, reviewed and edited the Draft; J.V. whose involvement in this project was advisory.

Conflicts of interest

The authors declare that there is no conflict of interest.

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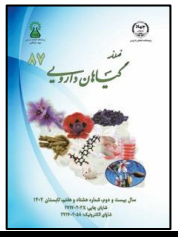

مقاله تحقیقاتی

ارزیابی کمی فلاونوئیدها با استفاده از تکنیک HPLC و ارزیابی فعالیت آنتی‌اکسیدانی در دو اندام *Salvia tebesana* Bunge

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اطلاعات مقاله

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مریم‌گلی

چکیده

مقدمه: مریم‌گلی به عنوان گیاهی مهم در طب‌سنتی، در نقاط محدودی از خاورمیانه پراکنده شده است. **هدف:** در این مطالعه سه حلال قطبی متانول ۸۰ درصد، اتانول ۸۰ درصد و آب دوبار تقطیرشده برای ترغیب ترکیبات فنلی *Salvia tebesana* مورد بررسی قرار گرفت. **روش بررسی:** در ابتدا فعالیت آنتی‌اکسیدانی (برآوردشده با ظرفیت آنتی‌اکسیدانی کل DPPH و سنجش مهار رادیکال FRAP) و محتوای فنل، ارتودی‌فنل، اسیدهای‌فنلی، فلاونوئید، فلاونول و پروآنتوسیانیدین عصاره‌های برگ و ساقه ارزیابی شدند. پس از انتخاب بهترین حلال، نمونه‌ها از نظر ترکیبات فلاونوئیدی مجزا (اپیژنین، کوئرستین، میریستین و روتین) توسط HPLC-PDA مورد سنجش قرار گرفتند. **نتایج:** عصاره‌های گیاهی مختلف فعالیت‌های قوی درمهار رادیکال‌ها نشان دادند و عصاره برگ به‌دست‌آمده با متانول ۸۰ درصد، بیشترین ظرفیت آنتی‌اکسیدانی را نشان داد. درهمین عصاره بیشترین محتوای ارتودی‌فنل، اسیدهای فنلی، فلاونوئید و پروآنتوسیانیدین به ثبت رسید (به ترتیب ۴۶/۲ ± ۱۵۹۸/۵، ۴۱/۳ ± ۷۴۲/۲، ۲۱/۹ ± ۴۸۷/۶ و ۳۱/۴ ± ۳۵۰/۱ میلی‌گرم در ۱۰۰ گرم ماده‌خشک)، درحالیکه عصاره اتانولی برگ بیشترین محتوای فنل و فلاونوئید را به خود اختصاص داد (به ترتیب ۴۷/۳ ± ۲۲۹۹/۲ و ۴۲/۳ ± ۳۵۹/۱ میلی‌گرم در ۱۰۰ گرم ماده خشک). سطح بیشینه فلاون اپیژنین (۱۱/۱ ± ۴۶۶/۱ میکروگرم در گرم ماده خشک) و فلاونول روتین (۱۰/۰۲ ± ۳۴۸/۶ میکروگرم در گرم ماده خشک) نیز در عصاره متانولی برگ یافت و برای اولین بار در *S. tebesana* گزارش شد. **نتیجه‌گیری:** این یافته‌ها تضمین می‌کند که *S. tebesana* به‌ویژه برگ‌ها، می‌تواند به عنوان یک آنتی‌اکسیدان طبیعی و در جهت کاهش تنش اکسیداتیو، در برنامه‌های غذایی و پزشکی پیشنهاد شود.

مخففها: BHA، بوتیلیتدهیدروکسی‌انیزول؛ BHA، بوتیلیتدهیدروکسی‌تولوئن؛ BTHQDPPH، ۲،۲-دی فنیل-۱-پیکریل‌هیدرازیل؛ EE، عصاره‌اتانولی؛ FRAP، توان آنتی‌اکسیدانی احیاء آهن، HPLC-PDA، کروماتوگرافی‌مایع با قدرت بالا؛ IC50، نیمه قدرت بازدارندگی بیشینه؛ ME، عصاره متانولی، PG ROS، گونه‌های کنشگراکسیژن، TAC، ظرفیت آنتی‌اکسیدانی‌کل؛ TPTZ، ۲،۴،۶- تری(۲-پیریدیل)-s-تریازین؛ WS، عصاره‌آبی

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