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Short Communication

Evaluation of Solvent Effect (Methanol: Water Mixture) on the Phenolic Content and Antioxidant Activities of *Stachys turcomanica* Trautv

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ABSTRACT

Background: The genus of *Stachys* with 300 species belongs to Lamiaceae family and this genus has 34 species in Iran. *Stachys turcomanica* Trautv is native to Iran and it used for treatment of infectious, rheumatic and inflammatory diseases in traditional medicine. The effect of solvent (methanol in water: 0, 20, 50, 80 and 100 %) on the extraction of antioxidant compounds from *S. turcomanica* were investigated using various *in vitro* assays.
Methods: The antioxidant activities of extracts were studied via three methods: FRAP (ferric reducing antioxidant power), DPPH (2, 2-diphenyl-1-picrylhydrazyl) and beta-carotene linoleic acid bleaching.
Results: The results of present work revealed that the solvent combinations have effects on the extraction of phenolic compounds and antioxidant properties. The relative methanol/water ratio of 80:20 v/v was effective solvent in the extracting of phenolic compounds. Also, there was a good correlation between antioxidant activity and total phenolic content *S. turcomanica* extracts.
Conclusion: The results demonstrated methanol/water (80:20) ratio was the best solvent to release of many secondary metabolites from *S. turcomanica* for future studies, which could provide natural sources of antioxidant compounds.

Introduction

The genus of *Stachys* with 300 species belongs to Lamiaceae family and it was found in Iran and Mediterranean area.[1] In Iran, this genus has 34 species.[2] The plants in this genus have anticancer, antipyretic, antitoxic, anti-anxiety, antiproliferative, anti-inflammatory, antibacterial and antioxidant activities.[3-9] Some species of this genus are used as disinfectant, antispasmodic and anti-fever agents.[10] Phytochemical analysis demonstrated presence of monoterpenes, sesquiterpenes,[11,12] flavonoids[13] and phenylethanoid glycosides in this genus.[14,15] In Iran, the aerial parts of *Stachys* genus are used for treatment of infectious, rheumatic and inflammatory disease.[16] One of the Iranian species is *Stachys turcomanica*.[2,17]
Medicinal plants are known for their antioxidant compounds and they are less toxic than synthetic antioxidants.[18,19] Some conditions such as temperature, type of solvent, concentration and extraction time have effects on the extraction of phenolic compounds.[20] Selection of solvent is important, because type of solvent and extracted compounds determine the antioxidant property of one extract.[21] Antioxidant activity in plants has been associated with phenolic compounds such as carotenoids, vitamins, phenols and flavonoids.[22,23] The objective of the present study was to evaluate the effects of solvents on the extraction of phenolic compounds and antioxidant activity of *S. turcomanica* by *in vitro* methods.

Material and Methods

Plant material

The aerial parts of *S. turcomanica* were collected in Jun 2016 from the North Khorasan Province Mountains of Iran. The plant was identified by Natural Products and Medicinal Plants Research Centre of North Khorasan University of Medical Sciences (Iran). Voucher number was MP264/N.Khorasan.

Preparation of plant extracts

About 150 g of plant was macerated in methanol-water (20, 50, 100 v/v) at room temperature for 48 h separately and then the solvent was removed under vacuum at 40 °C to give the crude extract.[24]

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Total phenolic determination

For measurement of total phenolic content, 100 µL of extract was added to 2.8 mL of distilled water, 2 mL of sodium carbonate (2%) and 100 µL of Folin-Ciocalteu reagent (50%) and then tubes were incubated for 30 min. After that, their absorbances were read at 720 nm compared to the control. For drawing the standard curve, Gallic acid was used as the standard. The total phenolic contents of the extracts were reported as milligrams of gallic acid per grams of dried weight of extract (mg GAE/g DW).[25]

Antioxidant activity assay

DPPH assay (2, 2-diphenyl-1-picrylhydrazyl)

In this test, antioxidant activity was described by free radical scavenging capacity of the extract based on one-electron reduction.[26] In this method, DPPH solution (0.004% w/v, 0.1 mM) was prepared in methanol, 0.1 mL of sample solution was added to 3.9 mL of DPPH solution and then incubated at room temperature for 30 min, the absorbance was read at 517 nm and the experiment was carried out in triplicate.[27] Methanol was used as blank, ascorbic acid and BHT as positive controls. The percentage of radical scavenging activity was calculated from Equation (1):

$$Percentage\ of\ inhibition = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \qquad \text{Eq. (1)}$$

Ferric reducing antioxidant power (FRAP assay)

100 µL of each extract was added to FRAP reagent (3 mL: sodium acetate buffer 300 mM ;pH 3.6, tripyridyl triazine (10 mM) and ferric chloride (20 mM)). The mixture was vortexed and incubated at 30°C for 4 minutes. The control was distilled water (100 µL) with FRAP reagent (3 mL). Absorbances of the solutions were read at 593 nm against control. Aqueous solutions of $FeSO_4.7H_2O$ (0-1 mM) were used for calibration curve. The calibration curve was plotted with absorbance and concentration, and then total antioxidant activity was expressed as mmol Fe (II)/ g extract (mean ± standard error).[28] In this method, the electron donating of antioxidant compounds at low pH cause to reduction of ferric to ferrous and they can convert colorless ferric tripyridyl triazine complex to blue ferrous tripyridyl triazine which has absorbance at 593 nm.

β-carotene Bleaching Assay (BCBA)

Antioxidant activity of *Stachys turcomanica* for inhibition of linoleic acid oxidation was studied by β-carotene linoleic acid method.[29] In this method, 200 mg of tween 20, linoleic acid (20 µL) and β-carotene (1 mg) were added to 5 mL of chloroform. After that the mixture was put at 40° C until the chloroform to evaporate. Then, 50 mL of distilled water was added to the mixture and it was shacked for 30 min. 50 µL of each extract was added to 6 mL of this mixture. In the control tube, the mixture was added to 50 µL of methanol. After that, the absorbance was measured at 470 nm (A_0), and the tubes were placed in a water bath at 50 °C for 2 h to catalyze the oxidation reaction and discoloring of β-carotene. The absorbance of sample was measured at 470 nm (A_{120}) to calculate the decreased absorbance in each sample. BHT was applied as positive control. All of the analyses were performed in triplicate. The antioxidant activity index (AAI) was carried out using Equation (2):

$$AAI\% = 1 - \frac{(A0 - A\,120)sample}{(A0 - A\,120)control} \times 100 \qquad \text{Eq. (2)}$$

Results and Discussion

Selection of solvent is the important step in optimizing the recovery of antioxidant compound from a sample. In this study, the effect of solvent type in total phenolic and antioxidant activity was evaluated. The yields of extraction were shown in Table 1. As shown in Table 1, the highest yield extraction was for methanol 80% with 27.33% yield and water extract had lowest yield of extraction. In other studies, effects of solvents in extraction of different polyphenols have been reported [30] and they showed efficiency of water and methanol as extraction solvents.

In this study, total phenolic compounds of extracts were evaluated and the results were presented in Table 1. The Folin-Ciocalteu method is a widespread assay for quantitative determination of phenolic content. In this method, the oxidation of phenolic compounds in alkaline solution was done.[31] The phenolic compounds donate electrons and delocalize the unpaired electron with their aromatic structures.[32] In this study, the 80% methanolic extract was found to contain higher total phenolic content than other extracts. Thus, this solvent is considered as a better and more efficient solvent for extraction of phenolic compounds.

Table 1. Extraction yield and total phenolic content of various extracts from *S. turcomanca.*

Extracts	Extraction yield (%)	Total phenolic contents* (mg GAE/kg DW)
Methanol 100%	21.24	3029.46±1.1
Methanol 80%	27.33	4197.8±3.5
Methanol 50%	21.5	3732.6±2.5
Methanol 20%	20.0	2380.5±1.5
Water	10.37	1299.0±1.7

*Results correspond to the average ± standard deviation estimated from three aliquots of extracts.

The use of natural antioxidants have few side effects because of their low toxicity compared to other drugs.[33] Several methods have been used for evaluation of antioxidant activity and each of them has some problems and limitations.[33,34] In this study, antioxidant properties of different extracts were

evaluated with three methods FRAP, DPPH and β-carotene bleaching.

The DPPH method is commonly method because it is simple, efficient and inexpensive. In this method, DPPH that is a free radical has a maximum absorption at 517 nm and DPPH scavenging activity is based on the ability of sample to donate hydrogen which reacts with the DPPH radical and a reduction in absorbance is happened.[35,36] The DPPH scavenging activity of extracts is dependent on concentration. In this test, the results are reported as IC_{50}, which is defined as the amount of antioxidant can inhibit 50% of DPPH free radicals. A lower value of IC_{50} indicates a higher antioxidant activity.[37] IC_{50} values of extracts and positive controls were shown in Table 2. In the present study all of extracts had lower antioxidant activities as compared to the standard BHT. Also, the strongest DPPH activity was obtained by 80% methanolic extract and lowest activity was obtained in 20% methanolic extract. In the FRAP assay, the ferric reducing ability was measured and the antioxidant activities were expressed by the reduction of ferric tripyridyltriazine complex to form a blue color ferrous tripyridyltriazine complex.[38] In the present study, the FRAP value expressed in Table 2. The results showed methanol 80% extract revealed the highest ferric reducing potential.

Table 2 show the antioxidant capacity of extracts using the beta-carotene/linoleic acid. The results showed that there are different amounts of antioxidants in various extracts and methanol 80% with 35.85% inhibition had highest antioxidant activity in this method. In this study, with increasing water content in the solvent, yield, antioxidant activities and total phenolic content were decreased. The total phenolic contents and antioxidant activities were decreased with increasing water solvent in extracting, because the water extract contain nonphenolic compounds or possess phenolic compounds that contain a smaller number of active groups than the other solvents.

Also in this study, the correlation coefficients between the antioxidant activity and total phenolic content were evaluated. Significant positive relationships were also obtained between total phenolic content and the antioxidant activities that evaluated by FRAP, DPPH, beta-caroten linoleic acid assays (R^2= 0.768, 0.692 and 0.550 respectively).

The results showed stronger correlation between antioxidant activities and total phenolic content; thus in this study, strong contributions of phenolic compounds to the antioxidant properties of *S. turcomanica* were shown.[39] Also these results showed, the β-caroten linoleic acid method was the least correlated with total phenolic content. This may be due to the electron transfer reaction mechanism in total phenolic content assay, FRAP and DPPH methods. In other studies this correlation was shown.[40]

Other studies were done on the *S. turcomanica*, such as in Firouznia research, the results showed the oil of *S. turcomanica* consists of thirteen monoterpene hydrocarbons (34.4 %), eleven sesquiterpene hydrocarbons (52.6 %) and four oxygenated sesquiterpenes (6.0 %). [41]

In khanavie et al work, the results showed ethyl acetate and chloroform fractions of *S. turcomanica* represented moderate cytotoxic activity against HT-29 cell line (IC_{50} < 70 µg/mL).[42] Khanavi et al have investigated antioxidant activity of some *Stachys* species from Golestan park of Iran, and their results demonstrated that the most of the strong antioxidants and phenolic compounds were soluble in methanol in *Stachys* species.[43]

Conclusion

Our results indicated that solvent combinations have effects on the extraction of phenolic compounds and antioxidant capacities. In addition, there were good correlations between antioxidant capacity and total phenolic contents of the *S. turcomanica* extracts. We introduce the methanol:water (80:20) extract was good solvent in extracting of phenolic compounds with highest antioxidant activity. These results demonstrated this extract was the best solvent to release of most secondary metabolites from *S. turcomanica* for future studies, which could provide natural sources of antioxidant compounds.

Table 2. Antioxidant activity of various extracts of *S. turcomanica* using the DPPH, FRAP and β-carotene/linoleate model systems.

Extracts	IC_{50} (mg/ml) in DPPH assay	FRAP value (mmolFe^{2+}/g dry extract)	β-Carotene–linoleate (% inhibition)
Methanol 100%	2.58±0. 41	1240±0.28	28.59± 0.51
Methanol 80%	1.71±0. 58	1330±0.36	35.85± 0.19
Methanol 50%	1.87±0.21	1260±0.51	33.9± 2.85
Methanol 20%	9.93±0.64	680±0.49	11.18± 0.15
water	6.98±0.37	960±0.34	29.6± 0.46
BHT	0.125±0.17	14.3±0.58	93.25± 0.09

Conflict of interests

The authors claim that there is no conflict of interest.

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