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Comparison of the Total Phenol, Flavonoid Contents and Antioxidant Activity of Methanolic Extracts of *Artemisia spicigera* and *A. splendens* Growing in Iran

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ABSTRACT

Background: The present study is designed to evaluate the radical scavenging activity, total phenol content (TPC) and total flavonoid content (TFC) of the MeOH extracts and their solid phase extraction fractions of *A. spicigera* and *A. splendens*. ***Methods:*** The antioxidant activity of the extracts and fractions were examined by DPPH method. Total phenol and total flavonoid quantities of the samples were determined spectrophotometrically using Folin-Ciocalteu and $AlCl_3$ reagents respectively. ***Results:*** The 40% MeOH-water fractions in both plants exhibited the highest degree of free radical scavenging property (RC_{50} 0.0094 ± 0.002, 0.0121 ± 0.003 mg/ml for *A. splendens* and *A. spicigera*, respectively) compared to that of the positive control quercetine 0.0039 mg/ml. As assumed, the amount of total phenolics was very high in 40% MeOH-water fractions (33.69 ± 1.49, 36.67 ± 2.26 mg GAE /100 g of dry extract of *A. spicigera* and *A. splendens*, respectively) and also this fraction has been found to be rich in flavonoids (96.41 ± 8.74, 129.80 ± 7.76 mg rutinoside per 100 g dry extract of *A. spicigera* and *A. splendens*, respectively). ***Conclusion:*** A positive result observed between the free radical scavenging activity potential and total phenol and flavonoid levels of samples.

Introduction

It has been reported that free radicals contribute to pathogenesis of many diseases in humans, like arthritis, atherosclerosis, emphysema,[1] diabetes[2] cancer,[3] cardiovascular and neurological disorders.[4] Application of synthetic antioxidants such as butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT), tertiary butylated hydroquinone and gallic acid esters, have been suspected to cause or prompt negative health effects[5] and are reported to cause liver disorders.[6] Therefore there is a growing interest day by day in the substances exhibiting antioxidants properties from botanical sources especially native herbs. The genus *Artemisia (Asteraceae)* consists of a variable number of species from 200-400 in the world[7,8] and it is represented 34 species in the flora of Iran with the common Persian name "Dermane".[9] Biological compounds such as Several coumarins, flavonoids, phenylpropanoids, sterols and terpenoids (specially sesquiterpenes and monoterpenes), and their glycosides have been isolated from this genus, and possess antimalarial, antiviral, antitumor, antipyretic, antihaemorrhagic, anticoagulant, antiinflammatory, antioxidant, antihepatitis, antiulcerogenic, antispasmodic and anticomplementary activities.[10] The objectives of this study were to investigate and comparison of the free radical scavenging activity of *A. spicigera* and *A. splendens* methanolic extracts and their fractions separately (I), determination of their phenol and flavonoid contents (II) and main chemical groups of natural compounds and finally find a relation between them (III).

Materials and Methods

Chemicals

Folin ciocaltea reagent and gallic acid were purchased from Fluka. DPPH was obtained from Sigma, Germany. All other solvents and chemicals were analytical grade.

Plant Material

The aerial parts of *A. spicigera* were collected from a place near the Aras river and Jolfa at E: 45° 17', N: 38° 39' (altitude of 700-750) at Eastern Azerbaijan province (Iran) during November 2009 and the aerial parts of *A. splendens* were collected from Kaleibar (gharedagh) at E: 46° 48', N: 38° 49' (altitude of 2300) at Eastern

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Azerbaijan province (Iran) during June 2010. The identity of the plants was confirmed by anatomical examination in comparison with the herbarium specimens (voucher Nos. Tbz-FPh 716, Tbz-FPh 717 for *A. spicigera* and *A. splendens* respectively) retained in the School of Pharmacy, Tabriz University of Medical Science, Iran.

Extraction and Fractionation

The dried and ground aerial parts of *A. spicigera* and *A. splendens* (120 g) were extracted with a Soxhlet apparatus with n-hexane, Dichloromethane (DCM) and methanol (MeOH), successively. A portion of the MeOH extract (2 × 2g) was subjected to solid-phase extraction (SPE) on Sep-Pak 10 g C18 cartridges (Waters, Ireland) with a step gradient of MeOH: Water mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0) as eluent. All extracts and fractions were separately concentrated using a rotary evaporator at a maximum temperature of 45 °C.

Free-Radical-Scavenging Activity

The ability of the extracts and fractions to scavenge radicals was assessed by the method is based on the reduction of DPPH (molecular formula C18H12N5O6) solutions in the presence of a hydrogen donating antioxidant. DPPH (8 mg) was dissolved in methanol (100 ml) to obtain a concentration of 80 µg/ml. The Methanol extracts and SPE fractions were dissolved in methanol to obtain a concentration of 1 mg/ml. Dilutions were made to obtain different concentrations of extracts and then diluted solutions (5 ml each) were mixed with DPPH (5ml). After a 30 minute incubation period at room temperature, the absorbance was read against a blank at 517 nm with a Shimadzu UV/Visible Spectrophotometer 160A (USA). The percentage reduction was plotted against the sample extract concentration in order to calculate RC50 values which is the extract concentration providing 50% loss of DPPH activity. Quercetine was used as positive control and all tests were carried out in duplicate.[11-13]

Total Phenol Content (TPC)

Total contents of the phenolic compounds in the MeOH extracts and its fractions were determined by the modified Folin- Ciocalteau assay[14] as gallic acid equivalents (GAE).[15] 1 ml of extracts samples (5 mg in acetone:water (60:40) v/v) were mixed with 0.2 ml Folin- Ciocalteau`s reagent (1:2 diluted with water) and 1 ml of 2% Na_2CO_3 was added to the mixture. As control, reagent without adding extract was used. After incubation of the samples at room temperature for 30 min, their absorbances were measured at 750 nm (Pharmacia biotech Ultrospec 2000, UV/Visible spenttrophotometer, England). For the calibration curve, 10 mg of gallic acid was dissolved in 10 ml of acetone:water (60:40) v/v as a stock solution. Different dilutions of were prepared and were determined by Folin- Ciocalteau`s method. Experiments were reported 3 times for every dilution and a calibration curve was created.

Total Flavonoid Content (TFC)

The flavonoid content of the MeOH extracts and their fractions were determined using a modified colorimetric assay[16] and used rutinoside as a standard. Extracts or standard solutions (0.5 ml) were mixed with distillated water (2 ml) and 5% $NaNO_2$ (150 µl). After standing for 6 minutes, mixer was combined with 10% $AlCl_3$ solution (150 µl), 4% NaOH (2 ml) and finally distillated water was added to make a volume of 5 ml in a 5 ml volumetric flask).After incubation of the samples at room temperature for 30 minutes, the absorbances of the samples was read at 510 nm against blank and the total flavonoid content was expressed as rutinoside equivalents in mg per 100g of dried extract.

NMR Spectra from Methanolic Extracts and Their Fractions

To determine the presence of different groups of natural compounds in the methanol extract and its fractions, HNMR spectra of them were recorded in CD_3OD on a Bruker 200 MHz NMR spectrometer. TMS was used as internal standard.

Statistical Analysis

All experiments were conducted in duplicate and triplicate measurements and presented as the mean ± standard deviations. Data were analyzed by Excel 2007 Microsoft. The IC_{50} values were calculated from linear regression analysis.

Results

The results of total phenolic contents, total flavonoid contents and antioxidant activity obtained for MeOH extracts of *A. spicigera* and *A. splendens* and their fractions are given in Table 1.

Free-Radical-Scavenging Activity

Antioxidant activity of the extracts and fractions determined by DPPH method is based on the ability of antioxidants to accept electron or hydrogen to become a stable diamagnetic molecule (Diphenylpicrylhydrazyl).[17] It was found that all extracts and fractions reduced DPPH radicals in a concentration-dependent manner. The lower RC_{50} values indicates a stronger ability of the antioxidant substance to scavenge the DPPH radicals while the higher RC_{50} values indicates a lower scavenging activity of the scavengers as more scavengers were required to achieve 50% scavenging reaction. Compared to the standard antioxidant Quercetine (RC_{50} 0.0039 mg/ml) both the crude MeOH extracts and their fractions exhibit moderate to strong radical scavenging activities (RC_{50} 0.0094-0.4531 mg/ml), but the free radical scavenging of 40% MeOH-water fraction (RC_{50} 0.0094, 0.0121 mg/ml for *A. splendens* and *A. spicigera*, respectively) and 60% MeOH-water fraction

(RC_{50} 0.0171, 0.0153 mg/ml for *A. splendens* and *A. spicigera*, respectively) were superior to that of the other fractions.

The radical scavenging activity in the plant extracts and fractions decreased in the following order (Figure 1):

A. spicigera 40% > 60% > 80% > 20% > 10% > MeOH> 100%

A. splendens 40% > 60% > 10% > MeOH > 80% > 20% > 100%

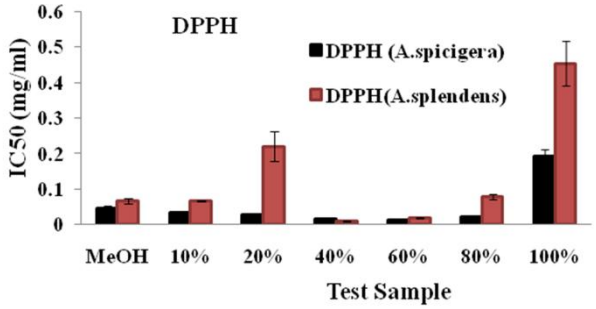

Figure 1. Antioxidant activity (IC50) of *A. spicigera* and *A. splendens* methanol extract and their solid phase extraction fractions. Note: antioxidant is expressed as the weight extracts and fractions required for 50% reduction in free radical generation. Therefore a lower value indicates a greater antioxidant activity. The values are reported as Mean ± SD.

Total Phenol and Total Flavonoid Contents

Total phenolic content was determined in comparison with standard Gallic acid and the results expressed in terms of mg GAE/ 100g dry sample in table 1. According to the results obtained from the determination of total phenolic contents, it was found that in both plants the 40% and 60% MeOH-water fractions contained more phenolic contents than the other fractions and MeOH extracts with 33.69, 36.67 mg GAE /100g of extract sample of 40% MeOH-water fraction and 31.20, 27.82 mg GAE /100g of extract sample of 60% MeOH-water fraction *A. spicigera* and *A. splendens*, respectively. Other fractions generally possessed low total phenolic contents with the range of 9.41-20.62 mg GAE /100g of extract powder (Figures 2, 3). Quantity of flavonoids in 40% and 60% MeOH-water fractions followed similar pattern. Total flavonoid content (TFC) was determined in comparison with standard rutinoside and the results expressed in terms of mg rutinoside per g dry sample. The TFC values for 40% and 60% MeOH-water fractions of *A. spicigera* and *A. splendens* were 96.41, 129.80 and 42.10, 4.44 mg rutinoside per 100 g dry sample respectively.

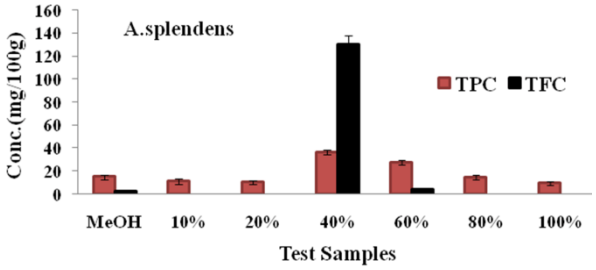

Figure 2. Comparison of total phenol (mg gallic acid/100g dry plant) and total flavonoid (mg rutinoside/100g dry plant) of MeOH extract and its solid phase extraction fractions of *A. splendens*. The values are reported as Mean ± SD.

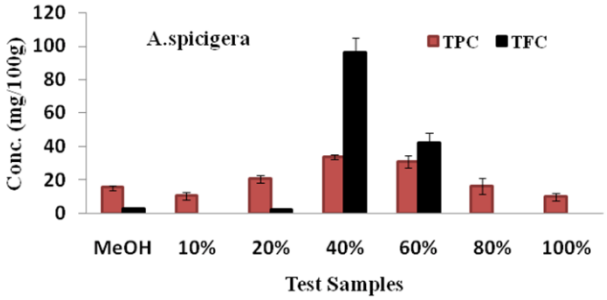

Figure 3. Comparison of total phenol (mg gallic acid/100g dry plant) and total flavonoid (mg rutinoside/100g dry plant) of MeOH extract and its solid phase extraction fractions of *A. spicigera*. The values are reported as Mean ± SD.

Table 1. Total Phenolic Contents (TPC), Total Flavonoid Content (TFC) and antioxidant activity of the MeOH extracts of *A . spicigera* and *A. splendens* and their fractions.

Extracts & Fractions	Total Phenol Content (TPC) mg/100g*		Total Flavonoid Content (TFC) mg/100g**		Antioxidant Activity (RC50) mg/l	
	A . spicigera	*A. splendens*	*A . spicigera*	*A . splendens*	*A. spicigera*	*A. splendens*
MeOH Extract	15.50 ± 1.33	15.06 ± 2.09	2.60 ± 1.20	2.35 ± 0.86	0.0458 ± 0.006	0.0662 ± 0.006
10% MeOH-water fraction	10.51 ± 2.47	10.97 ± 2.22	0	0	0.0337 ± 0.002	0.0651 ± 0.002
20% MeOH-water fraction	20.62 ± 2.46	10.35 ± 1.35	2.35 ± 0.23	0	0.0256 ± 0.005	0.2200 ± 0.042
40% MeOH-water fraction	33.69 ± 1.49	36.67 ± 2.26	96.41 ± 8.74	129.80±7.76	0.0121 ± 0.003	0.0094 ± 0.002
60% MeOH-water fraction	31.20 ± 3.68	27.82 ± 1.93	42.10 ± 6.09	4.44 ± 0.72	0.0153 ± 0.000	0.0171 ± 0.001
80% MeOH-water fraction	16.41 ± 5.00	14.72 ± 1.69	0	0	0.0220 ± 0.003	0.0780 ± 0.007
100% MeOH-water fraction	10.07 ± 2.26	9.41 ± 1.52	0	0	0.1910 ± 0.020	0.4531 ± 0.062
Quercetine	-	-	-	-	0.0039	

*Experiment was performed in triplicate and expressed as mean ±SD
** Experiment was performed in duplicate and expressed as mean ±SD

Discussion

Several studies have shown that there is a positive correlation between total phenol contents and antioxidant activity of the plants material.[18-20] Flavonoids, including flavonols, flavones and condensed tannins, are a class of plant phenolics, which contain hydroxyl groups, are responsible for the radical scavenging and chelating properties.[1,21] According to our findings, proportion of flavonoids in total phenol of 40% and 60% MeOH-water fractions was higher than the other fractions and in parallel to this the free radical scavenging of these fractions were stronger to that of the others. It has been reported that the antioxidant ability of flavonoid molecules with polyhydroxylated substitution on rings A and B, is related to their ability to donate hydrogen atoms and thereby scavenge the free radicals produced during lipid peroxidation.[16,21] The results presented above in table 1 showed that the contents of the phenolic compounds in 10% and 100% MeOH-water fractions of both plants are close to each other (10.51, 10.97 mg/100g GAE of extract sample for 10% MeOH-water fraction and 10.07, 9.41 mg GAE /100g of extract sample for 100% MeOH-water fraction *A. spicigera* and *A. splendens*, respectively) but 10% MeOH-water fraction has been found to have stronger antioxidant activity in comparison to 100% MeOH-water fraction (IC_{50} 0.0337, 0.0651 mg/l for 10% MeOH-water fraction, 0.191, 0.453 mg/l for 100% MeOH-water fraction of *A. spicigera* and *A. splendens*, respectively). This may be explained by the fact that different types of phenolic compounds possess different antioxidant capacities which is related to their chemical structure. For example, the previous researches showed that phenolic compounds with ortho- and para- dihydroxylation or a hydroxy and a methoxy group or both have stronger antioxidant activity than simple phenolics[19] and also the presence of double bond conjugated and ketone groups in the whole molecule might play different polarities in the structure of the antioxidants and can be attributed to their antioxidant activity.[22] The other factor may be lead to this results, is related to the sensitivity of Folin-Ciocalteu reagent to a broad range of phenolic compounds whereas the DPPH free radicals show different sensitivity to various antioxidants. The Folin-Ciocalteu reagent react both free phenolics and bound phenolics in extracts and other samples, but the DPPH assay just determined free antioxidants and phenolics.[23] Therefore if the bound phenolics and antioxidants exist in 100% MeOH-water fractions, may not contribute radical scavenging activity in the DPPH assay. The results of ^{1}HNMR spectroscopy are parallel with these findings, too (Table 2). Fractions which show peaks in aromatic regions at δ_H 6-8 ppm, possess polyphenolic compounds and lower RC_{50} values. The ^{1}HNMR spectera of 10%, 80% and 100% MeOH-water fractions belonging to *A. spicigera* have revealed that there is no flavonoid compounds in these fractions but some other phenols may exist in low concentration in these fractions that cause antioxidant activity. Obviously the spectrums showed that flavonoid compounds exist in high concentrations in 40% and 60% MeOH-water fractions of both of plants. Moreover previous studies have shown that different types of chemical constituents were found in various species of *Artemisia* genus. There are mainly phenolics such as flavonoids[24-28] cinnamic acid derivatives[29,30] and coumarines.[13,31,32] Then further studies are needed for the isolation and elucidation of the structure of phenolic components and also more investigations are necessary for better understanding of their mechanism of action as antioxidants.

Table 2. Prediction of main chemical groups of natural compound in the MeOH extracts of *A . spicigera* and *A. splendens* and their fractions based on ^{1}HNMR spectra.

Extracts & Fractions	Predicted Compounds	
	A. spicigera	***A. splendens***
MeOH Extract	Mixture of phenols, sugars and aliphatic derivatives	Mixture of phenols, sugars and aliphatic derivatives
10% MeOH-water fraction	Sugar and aromatic compounds	Sugar and aromatic compounds
20% MeOH-water fraction	Flavonoid and methoxylated flavonoid glycosides	Cinnamic acid derivaties
40% MeOH-water fraction	Flavonoid and other phenolic glycosides	Flavonoid glycosides
60% MeOH-water fraction	Flavonoids (Aglycones)	Flavonoids (Aglycones) Cinnamic acid derivatives
80% MeOH-water fraction	Phenolic glycosides with aliphatic chains	Phenolic glycosides with aliphatic chains
100% MeOH-water fraction	Not defined	Not defined

Conclusion

The results presented above demonstrated that some fractions (40% and 60%) of MeOH extracts of these *Artemisia* species had moderate to strong antioxidant activity and it is possible to conclude that there is a positive relation between phenolic and flavonoid composition and antioxidant activity. Also based on the results of the current study and on recent literature data,[33] the conclusion can be drawn that fractionation of extracts and running their ^{1}HNMR could be valuable method for predicting of groups of natural compounds and interfacing of chemical and biological assessments. By the way, investigations are in process to

identification of the structure of these phenolics and flavonoids.

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