

In vitro antioxidant activities of aqueous and methanolic extracts of *Smyrniium cordifolium* Boiss and *Sinapis arvensis* L.

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Abstract

Recently, interest in plant-derived food additives has grown mainly because synthetic antioxidants suffer from several drawbacks. Some plants are traditionally used for both food and medicine in Iran. Aqueous and methanolic extracts of *Smyrniium cordifolium* Boiss leaves and stems and *Sinapis arvensis* L leaves, stems and flowers were prepared and evaluated for their total phenolic content (TPC), total flavonoids (TF) and antioxidant activity. Antioxidant activities were evaluated by five different methods: free radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), inhibition of lipid peroxidation and reducing power. It was found that the leaves of *S. cordifolium* had the highest antioxidant activities (TPC: 51.31 ± 1.06 mg gallic acid equivalents/g dry weight, DPPH: 78.25 ± 0.69 % inhibition, FRAP: 381.17 ± 7.35 $\mu\text{mol Fe}^{2+}$ /g dry weight, TEAC: 96.27 ± 0.20 $\mu\text{mol trolox equivalents/g dry weight}$ and TF: 19.00 ± 0.30 mg catechin equivalents/g dry weight) and thus could be rich sources of natural antioxidants. These results suggest that leaves of *S. cordifolium* may serve as a potential source of natural antioxidant for food and pharmaceutical application. Further research is needed to isolate, characterize, and identify the bioactive compounds present in these plants.

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Introduction

Many plants contain natural antioxidants that act in metabolic response to the endogenous production of free radicals and other oxidant species. These responses are due to ecological stress or are promoted by toxins produced by pathogenic fungi and bacteria (Mata *et al.*, 2007). Free radicals can attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury associated with degenerative diseases (Amarowicz *et al.*, 2004). Compared to other tissues, the brain is especially vulnerable to oxidant damage because its membrane lipid composition is enriched in oxidizable polyunsaturated fatty acids and because of its high oxygen consumption (Slotkin and Seidler, 2010).

The most widely used synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy (BHT) are quite volatile and decompose easily at high temperatures. There are some serious problems concerning the safety and toxicity of them related to their metabolism and possible absorption and accumulation in body organs and tissues (Abdalla and Roozen, 1999). Although the mammalian body has certain defense mechanisms to combat and reduce oxidative damage, epidemiological evidence indicates that the consumption of natural antioxidants such as flavonoids and other polyphenolics is advantageous

for our health. Therefore, the search for preparations of useful natural antioxidant is highly desirable. Flavonoids and other plant phenolics are especially common in the leaves, flowering tissues and woody parts, such as stems, bark and roots of plants. The antioxidant activity of these phenolics is mainly due to their redox properties, which allow them to act as reducing agents or hydrogen-atom donors. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Balasundram *et al.*, 2006).

Edible plants are important constituent of traditional diets in Iran. *Smyrniium cordifolium* Boiss and *Sinapis arvensis* L. stems are eaten raw. *Sinapis alba*, also known as *Brassica alba*, is an economically important plant of Brassicaceae, s. Commonly known as yellow or white mustard, and growing well in hot and dry environments (Abbasi *et al.*, 2011). *Smyrniium cordifolium* Boiss is used in treatments for internal organ edema, especially in bladder and kidney problems (Mehrabi and Mehrabi, 2011). However, there is limited information about these plants. This information is necessary to validate the safety, traditional uses, and may be used to establish antioxidant databases and find out new potential sources of natural antioxidants. The purposes of this

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study were to determine total phenolics and total flavonoids and to evaluate antioxidant activities of two edible plants using different *in vitro* assays. Furthermore, the efficiency of extraction by boiling water and methanol was compared.

Materials and Methods

Chemicals

2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), Folin-Ciocalteu (FC) reagent and gallic acid were purchased from Merck. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and catechin were purchased from Sigma-Aldrich. Linoleic acid was purchased from Fluka.

Plant samples and extraction

Fresh plant parts (flower, leaves and stems) of both *Smyrnum cordifolium* Boiss and *Sinapis arvensis* L were collected from Ilam province of Iran. Local names of these plants are Khartal and Vanegi, respectively. Samples were washed, dried and carefully powdered and stored at -20 °C in darkness.

For water extraction, 0.2 g of the fine powder was extracted with 10 ml of distilled water at 100°C for 1 h in a water bath. For methanolic extraction, 0.2 g of the powder was extracted with 10 ml of methanol (80%) at 60°C for 1 h. The samples were then centrifuged at 4500 rpm for 15 min and supernatants were used for analysis.

Total phenolic content

The total phenolic contents (TPC) of the extracts were determined using the Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Forty microliters of properly diluted extract solution were mixed with 1.8 ml of FC reagent. The reagent was also diluted 10 times with distilled water. The mixture was kept for 5 min at room temperature, then 1.2 ml of (7.5% w/v) sodium carbonate solution were added. The solution was mixed and allowed to stand for 1 hour at room temperature. Finally, the absorbance was measured at 765 nm using a UV-Vis spectrophotometer. A calibration curve was prepared using standard solutions of gallic acid. The results were expressed as mg gallic acid equivalents per g dry weight.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The radical scavenging activity of extract was measured by slightly modified method of Brand-Williams (Jaitak *et al.*, 2010). 2.9 ml of DPPH

radical solution (100 µM) prepared in 70% aqueous ethanol were added to 100 µl of extract. The mixture was shaken vigorously and allowed to stand at 25°C in dark for 30 min. Decrease in absorbance of the resulting solution was measured at 517 nm against a blank consisting of 100 µl of 70% aqueous ethanol and 2.9 ml of DPPH solution. All samples were analyzed in triplicate. Inhibition percentage of DPPH radical was calculated

$$\text{Inhibition\%} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay is based on the ability of a compound to scavenge the stable ABTS radicals (Arts *et al.*, 2003). The stock solutions included 7.0 mM ABTS solution (A) and 140 mM potassium persulphate (K₂S₂O₈) solution (B). For ABTS radicals production, the working solution was prepared by mixing 5.0 ml of A and 88 µl of B, and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml ABTS solution with 50 ml methanol. Fresh ABTS solution was prepared for each assay. Sample (200 µl) was mixed with 3.0 ml of ABTS solution and absorbance was then measured at 734 nm using the UV-Vis spectrophotometer. The antioxidant capacity was expressed as µmol trolox equivalents (TE) / g dry weight.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out according to the procedure of Benzie and Strain with slight modification (Benzie and Strain, 1996). The FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared daily and was warmed to 37°C in a water bath prior to use. Fifty microliters of sample were added to 1.5 ml of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. The standard curve was constructed using iron (II) sulfate solution, and the results were expressed as µmol Fe (II)/g dry weight of plant material.

Reducing power assay

The reducing power of extract was measured according to the method reported by Yen and Duh with some modification (1993). 2.5 ml of extract were mixed with 2.5 ml sodium phosphate buffer (pH = 6.6, 2.0 M) and 5.0 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for

20 min. Trichloroacetic acid (10%, 5.0 ml) was added to mixture, which was then centrifuged at 4500 g for 10 min. The upper solution (5.0 ml) was mixed with distilled water (5.0 ml) and 1.0 ml of ferric chloride (1.0%). The absorbance was measured at 700 nm and reducing power was expressed as trolox equivalents.

Lipid peroxidation inhibition

In a vial with a screw cap, four milliliters of extract solution were mixed with 4.1 ml linoleic acid in absolute ethanol (2.51%), 8 ml phosphate buffer (pH = 7.0, 0.05 M) and 3.9 ml distilled water. The mixture was then placed in an oven at 40°C for 16 h. Then, 75% ethanol (9.7 ml) and 30% ammonium thiocyanate (0.1 ml) were added to above mixture (0.1 ml). Three minutes after adding of 0.1 ml ferrous chloride (0.02 M in 3.5% hydrochloric acid) to the reaction mixture, the absorbance of red color was measured at 500 nm (Kikuzaki and Nakatani, 1993).

Determination of total flavonoid content

Total flavonoid content of extract was also determined (Jia *et al.*, 1998). Briefly, 250 µl of extract solution were diluted with 1.25 ml distilled water and then 75 µl of 5% NaNO₂ solution were added. The mixture was allowed to stand at room temperature for 6 min and then, 150 µl of 10% AlCl₃ were added. This mixture was allowed to stand for a further 5 min before 0.5 ml of 1 M NaOH was added. The solution was shaken vigorously and absorbance at 510 nm was measured with a UV-Vis spectrophotometer. The results were expressed as mg catechin equivalents per g of dry weight.

Statistical analysis

Pearson correlation coefficients were calculated to obtain the possible correlations. All calculations were performed using Minitab software (version 14.1, Minitab Inc., State College).

Results and Discussion

Total phenolic content

The phenolic contents of the aqueous and methanolic extracts of plants were tested using the diluted Folin-Ciocalteu reagent (FCR). Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH = 10). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. The reaction occurs through electron transfer mechanism. The blue compounds formed between phenolate and FCR are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination

complexes formed between the metal center and the phenolic compounds. It is believed that FCR contains heteropolyphosphotunstates-molybdates. Sequences of reversible one or two-electron reduction reactions lead to blue species, possibly (PMoW₁₁O₄₀)⁴⁻. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI) (Huang *et al.*, 2005). As shown in Table 1, there was large variation in the total phenolic contents of these plants, ranged from 5.04 ± 0.24 to 51.31 ± 1.06 and from 4.79 ± 0.30 to 22.26 ± 0.18 mg GAE/g dry weight for aqueous and methanolic extracts, respectively. The highest contents of total phenolics were observed for leaves of *S. cordifolium* (51.31 ± 1.06 mg GAE/g), followed by flowers and leaves of *S. arvensis* (15.76 ± 0.56 and 9.47 ± 0.41 mg GAE/g, respectively). The stems of *S. arvensis* had the lowest phenolics content (5.04 ± 0.24 mg GAE/g). A similar pattern was also observed in methanolic extracts.

Antioxidant capacity

The extracts of these edible plants were tested for antioxidant activity using different *in vitro* assays. DPPH radical-scavenging activity test measures the capacity of the extracts to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl. If the extracts have this capacity, the initial blue/purple solution will change to a yellow color due to the formation of diphenyl picrylhydrazine. The lowest DPPH radical scavenging of methanolic extracts were obtained by leaves of *S. arvensis* (12.31 ± 1.55%) and the highest DPPH radical scavenging were obtained by aqueous extracts of *S. cordifolium* Bois leaves (78.25 ± 0.69 %).

TEAC assay is more versatile as both the polar and non-polar samples can be evaluated for their scavenging activity. The spectral interference is minimized since the absorption maximum used is around 734 nm. In TEAC assay, the scavenging of free radicals was measured as the discoloration of the ABTS blue reactant. The values ranged from 70.11 ± 2.12 to 98.75 ± 0.39 and from 93.01 ± 2.22 to 98.89 ± 0.26 µmol TE/g dry weight for aqueous and methanolic extracts, respectively. Highest levels of antioxidant activity were obtained from methanolic extracts of leaves of *S. cordifolium* and the lowest was obtained from the aqueous extracts of stems *S. arvensis*.

FRAP assay is a simple assay that gives fast and reproducible results. The results were expressed as µmol ferrous iron equivalents per g of sample. Table 1 shows a wide range of the antioxidant activity of aqueous and methanolic extracts of edible plants. For

Table 1. Phenolic and flavonoid contents and antioxidant activity of aqueous and methanolic extracts

Samples	TPC ^a	DPPH (%)	TEAC ^b	FRAP ^c	FRP ^d	TF ^e	ILP ^f (%)
Aqueous							
<i>S. arvensis</i>							
leaf	9.47±0.41	24.72±0.44	97.70±0.22	56.70±3.60	35.24±0.01	2.54±0.16	36.64±5.30
flower	15.76±0.56	43.78±2.10	98.22±0.01	70.77±3.62	88.50±6.03	1.62±0.11	30.86±4.00
stem	5.04±0.24	21.87±0.69	70.11±2.12	41.37±0.58	15.66±1.00	3.05±0.03	31.96±1.56
<i>S. cordifolium</i>							
leaf	51.31±1.06	78.25±0.69	96.27±0.20	381.17±7.35	424.21±6.92	19.00±0.30	44.30±6.42
stem	5.78±0.15	25.44±0.13	98.75±0.39	33.63±1.34	37.91±0.56	1.35±0.24	17.70±2.82
Methanolic							
<i>S. Arvensis</i>							
leaf	9.76±0.33	12.31±1.55	98.22±1.28	61.70±5.41	44.41±0.70	6.01±0.22	16.26±0.97
flower	18.96±0.42	73.27±6.10	98.84±0.01	90.80±0.01	102.68±3.17	7.75±0.41	47.57±6.99
stem	4.79±0.30	23.94±2.77	93.01±2.22	33.8±3.39	25.05±0.25	3.67±0.46	51.86±0.72
<i>S. cordifolium</i>							
leaf	22.26±0.18	71.91±5.02	98.89±0.26	115.80±6.55	312.40±10.30	20.77±2.05	20.09±4.49
stem	6.61±0.34	24.22±1.07	95.48±5.65	37.44±2.18	62.92±1.57	5.78±0.23	17.50±0.82

^aTP: total phenolic content, mg/g dry weight in gallic acid equivalent.

^bµmol trolox equivalent/g dry weight.

^cµmol Fe²⁺/g dry weight of plant material.

^dFRP: ferric reducing power, µmol trolox equivalent /g dry weight.

^eTF: total flavonoid content, mg/g dry weight in catechin equivalent.

^fILP: inhibition of lipid peroxidation.

the aqueous extracts, the antioxidant activity ranged from 33.63 ± 1.34 to 381.17 ± 7.35 µmol Fe²⁺/g. Leaves of *S. cordifolium* had the highest antioxidant activity (381.17 ± 7.35 and 115.80 ± 6.55 µmol Fe²⁺/g for aqueous and methanolic extracts, respectively). Stems of *S. arvensis* had the lowest antioxidant activity.

Reductive capabilities of plant extracts can serve as a significant indicator of their potential antioxidant activities. The potassium ferricyanide reduction method is a widely used method for evaluating the ferric reducing power (FRP) of plant polyphenols. In this assay, antioxidants in test samples reduced the ferricyanide complex to the ferrous form by donating an electron. Fe³⁺ reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action. The reducing power was ranged from 15.66 ± 1.00 to 424.21 ± 6.92 µmol trolox equivalents per g dry weight for aqueous extracts. Leaves of *S. cordifolium* and stems *S. arvensis* had the highest and lowest reducing power, respectively.

Total flavonoids

It has been recognized that phenolic contents of botanical materials are associated with their antioxidant activities. Flavonoids found ubiquitously in plants are the most common group of

phytophenolics. Flavonoids content was determined using the aluminium chloride colorimetric method. The results were expressed as mg catechin per g dry weight. Catechin was used as standard in the same conditions. There was large variation in the total flavonoid content, ranging from 1.35 ± 0.24 to 19.00 ± 0.30 and from 3.67 ± 0.46 to 20.77 ± 2.05 mg CE/g dry weight for aqueous and methanolic extracts, respectively. The highest total flavonoid content of aqueous/methanolic extracts was obtained from leaves of *S. cordifolium*. The lowest level of total flavonoid contents was obtained from the aqueous extracts from stems of *S. cordifolium*.

Inhibition of lipid peroxidation

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specifically, linoleic acid and arachidonic acid are targets of lipid peroxidation. The inhibition of lipid peroxidation by antioxidants is due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids. In this assay, methanolic extracts of *S. arvensis* showed the most inhibition against lipid peroxidation (Table 1). There are various types of phytochemicals, including flavonoids, coumarins,

xanthenes, phenylpropanoids and terpenoids, which are effective in preventing of lipid peroxidation.

Correlation between total phenolic content and antioxidant capacity

The correlation coefficients (R^2) between the total phenolic content and the antioxidant activities of these plants were also determined. There were high positive linear correlations between total phenolic content and antioxidant activities for aqueous ($R^2_{\text{TPC, DPPH}} = 0.966$, $R^2_{\text{TPC, FRAP}} = 0.983$, $R^2_{\text{TPC, FRP}} = 0.992$ and $R^2_{\text{TPC, TF}} = 0.930$) and methanolic extracts ($R^2_{\text{TPC, DPPH}} = 0.829$, $R^2_{\text{TPC, FRAP}} = 0.978$, $R^2_{\text{TPC, FRP}} = 0.707$ and $R^2_{\text{TPC, TF}} = 0.684$). This result indicates phenolic compounds are the major contributor of antioxidant activities of these plants. Each antioxidant assay only provides an estimate of antioxidant capacity that is subjective to its conditions and reagents. Therefore, the use of different methods helps to identify variations in the response of the extracted compounds.

Conclusion

Recently, interest has increased in naturally-occurring antioxidants that can be used to protect human beings from oxidative stress damage. In food science, antioxidants are very important in that they act preventing lipid oxidation in food and decreasing the adverse effects of reactive species on normal physiological functions in humans. In this study, antioxidant capacities of the aqueous and methanolic extracts of two edible plants from the western of Iran were determined by seven *in vitro* assays. All extracts exhibited antioxidant activity, the best results being obtained with the aqueous extracts of leaves of *Smyrniium cordifolium* Boiss and it was found to be valuable sources of natural antioxidants, both for preparation of crude extracts and for further isolation and purification of antioxidant components. A significant relationship between the antioxidant capacities and total phenolic contents were found and phenolic compounds were the major contributor of antioxidant activities of these plants. After this comparative study, it is suggested that further work is needed to isolate, characterize and identify the bioactive compounds present in these plants.

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