

Antioxidant activity of bulbs and aerial parts of *Crocus caspius*, impact of extraction methods

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Abstract: *Crocus* genus (*Iridaceae*) is comprises approximately 80 species. In this study *in vitro* antioxidant activities of extracts from *C. caspius* bulbs and aerial parts were investigated. Ultrasonically assisted extraction (US), percolation method (PE) and polyphenolic fraction (PP) were used. Antioxidant activities were evaluated with five different tests. Aerial parts US extract with high levels of phenol and flavonoids were the most potent extract in DPPH radical scavenging than others. Aerial parts PE extract had shown very potent reducing power, which was so better than other extracts ($p < 0.01$). Aerial parts PP fraction showed very good Fe^{2+} chelating ability. Aerial parts US extract were the most potent extract in scavenging of H_2O_2 . Bulb PP fraction with $\text{IC}_{50} = 22.8 \pm 0.7 \mu\text{g ml}^{-1}$ was the most potent fraction in nitric oxide scavenging. The results improved high levels of antioxidant activities of *C. caspius* bulbs and aerial parts in all tested models.

Keywords: Antioxidant activity; *Crocus caspius*; radical scavenging; ultrasonic extraction; polyphenol fraction.

INTRODUCTION

When body cells make use of oxygen for generation of energy, excessive free radicals are generated due to unbalance oxidants and antioxidants ratio. They are generally reactive oxygen species (ROS). These species generate oxidative stress and play very important role in the progression or initiation of some diseases (Valko *et al.*, 2007). Antioxidants scavenge and control the formation of free radicals and prevent damages caused by ROS. They can enhance body defense mechanisms and lower the risk of many diseases such as cancer and degenerative. In recent years, great attention has been paid to antioxidant properties of natural products, which may be used for human consumption. Among the various medicinal plants, some endemic species are of particular interest because they may be used for producing phytochemicals with significant antioxidant capacities and health benefits (Ebrahimzadeh *et al.*, 2010a). Phytochemical such as phenolics and flavonoids have antioxidant activities and can be used for scavenging of excess free radicals present in the body (Pietta, 2000).

Crocus genus (*Iridaceae*) is comprises approximately 80 species distributed from Europe to Asia (Mathew, 1982; Alavi-Kia *et al.*, 2008). The *Crocus* genus is known mainly for the valuable aromatic and medicinal cultivated species *C. sativus* (saffron), which is of great economic importance (Beiki *et al.*, 2010). Saffron has been used as a drug in folk medicine, particularly in traditional Indian folk medicine, where it has been used for the treatment of various kinds of mental illnesses among other uses without any toxic side effects (Abdullaev, 1993). Modern pharmacological studies have shown that oral

administration of saffron in humans was equally effective with imipramine and fluoxetine in the treatment of mild to moderate depression (Noorbala *et al.*, 2005) and in mice reversed the loss of learning and memory (Abe and Saito, 2000). Saffron also inhibited $\text{TNF}\alpha$ -induced apoptosis of PC12 cells that have been differentiated into neurons (Soeda *et al.*, 2001). Antioxidant properties of *C. sativus* stigmas extract (by FRAP and TEAC assay) and its anti-amyloidogenic activity have been reported (Papandreou *et al.*, 2006). In this study, *in vitro* antioxidant activities of *Crocus caspius* bulbs and aerial parts were investigated. Ultrasonically assisted extraction and percolation method were used as extraction methods. Nothing was found in literature about antioxidant activity of this plant. Also, these total extracts were compared with one biologically active fraction, i.e. polyphenolic fraction. Studies included 1,1-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide and nitric oxide radical scavenging activities, iron II chelatory capacity and reducing power. The total phenolic and total flavonoid contents also were investigated. This study is an attempt to report the antioxidant activity of *C. caspius* for the first time.

MATERIALS AND METHODS

Chemicals

Ferrozine, Trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH) and Hydrogen peroxide were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Quercetin, Gallic acid, EDTA, Potassium ferricyanide, Ascorbic acid, Butylated hydroxyanisole (BHA), and Ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used were of the highest commercially available purity.

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Plant material and preparation of freeze-dried extract

Crocus caspius bulbs and aerial parts were collected from Gele-Cola, Sari, Mazandaran, Iran and identified by Dr. Bahman Eslami. A voucher specimen (No 742) has been deposited in Sari School of Pharmacy herbarium. The parts of interest were dried at room temperature and coarsely ground (2-3mm) before extraction. Each part (100g) separately was extracted by percolation using methanol for 24 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper, repeated three times. The resultant extracts were concentrated in a rotary evaporator until a crude solid extracts were obtained which was then freeze-dried for complete solvent removal (PE extract).

Preparation of polyphenol fraction

Polyphenols were extracted from bulbs and aerial parts, according to our recently published paper (Rabiei *et al.*, 2012). The extraction was performed twice at 20°C in a shaking incubator. Extracting time was 30min and extracting solvent was 100ml of methanol/acetone/water (3.5/3.5/3) containing 1% formic acid. All extracts were collected and evaporated under vacuum at 35-40°C to remove methanol and acetone. Lipophilic pigments were then eliminated from the aqueous phase by extraction with petroleum ether. The aqueous phase was collected and further extracted three times by ethyl acetate. Organic phases were collected and concentrated over a rotary vacuum until a crude solid extract was obtained, which was then freeze-dried for complete solvent removal and used as polyphenol (PP) fraction.

Ultrasonically assisted extraction

Bulbs and aerial parts were extracted with methanol in an ultrasonic cleaning bath over a one hour by indirect sonication at a frequency of 100kHz and a temperature of 25±3°C to yield ultrasonic extract. The extract was then separated from the sample residue by filtration. The resultant extract was concentrated in a rotary evaporator until a crude solid extract was obtained which was freeze-dried for complete solvent removal and used as ultrasonic (US) extract (Rabiei *et al.*, 2012).

Determination of total flavonoid content

Colorimetric aluminum chloride method was used for determination of flavonoid (Ghasemi *et al.*, 2009). 0.5mL solution of each extracts (in methanol) were separately mixed with 1.5mL of methanol, 100µL of 10% aluminum chloride, 100µL of 1M potassium acetate, and 2.8mL of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Total flavonoid contents were calculated as quercetin equivalent from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100mg ml⁻¹ in methanol.

Determination of total phenol content

Total phenolic content was measured by colorimetric method using Folin-Ciocalteu reagent (Ghasemi *et al.*, 2009). The extract samples (0.5ml) were mixed with Folin Ciocalteu reagent (5 ml, 1: 10 diluted with distilled water) for 5min and aqueous Na₂CO₃ (4ml, 1M) were then added. The absorbance of the reaction mixture was measured at 760 nm after incubation for 2h at room temperature. Gallic acid was used as a reference standard. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml⁻¹ solutions of gallic acid in aqueous methanol (50%). The results were expressed as milligram gallic acid equivalent (mg GAE)/g of extract.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (Ebrahimzadeh *et al.*, 2009a, 2010b). 2ml of each extracts (with different concentrations) were added to 2ml of methanolic solution of DPPH (100µM). After 15 min at room temperature in the dark, the absorbance was read at 517 nm. The experiment was repeated for three times. Percentage of inhibition was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A₀ was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Vitamin C, BHA and quercetin were used for comparison. IC₅₀ values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Reducing power determination

The reducing power of extracts were determined according to our recently publish paper (Nabavi *et al.*, 2010). Different amounts of each extracts in water were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). After incubation of the mixture for 20min at 50°C, 2.5ml of trichloroacetic acid (10%) was added to stop the reaction. The mixture was centrifuged at 3000rpm for 10min. 2.5ml of the resulting supernatant was mixed with an equal volume of distilled water, and FeCl₃ (0.5ml, 0.1%). The absorbance was measured at 700nm. Vitamin C was used for comparison.

Metal chelating activity

The chelating of ferrous ions by extracts was estimated by our recently published paper (Ebrahimzadeh, *et al.*, 2009b). 1ml of different concentrations of extracts were added 2.8ml of distilled water and then mixed with 50µl of 2mM FeCl₂ and 150µl of ferrozine (5mM). The mixture was shaken vigorously and left standing at room temperature for 10min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine- Fe²⁺ complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A₀ was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Na₂EDTA was used as positive control.

Table 1: Phenol and flavonoids contents and antioxidant activities of *Crocus caspius* bulbs and aerial parts

<i>Crocus caspius</i>	Yield %	Total phenolic contents (mg g ⁻¹)	Total flavonoid Contents (mg g ⁻¹)	DPPH free radical scavenging, IC ₅₀ (μg ml ⁻¹) ^a	H ₂ O ₂ scavenging activity, IC ₅₀ (μg ml ⁻¹) ^b	Fe ²⁺ chelating ability, IC ₅₀ (μg ml ⁻¹) ^c	NO scavenging activity, IC ₅₀ (μg ml ⁻¹) ^d
Bulb							
Percolation	14	58.2±1.8	11.8±0.7	885.2±16.7	420.9±17.3	313.5±12.7	954.9±26.8
Ultrasonic extract	10	54.3±1.5	11.6±0.4	906.1±21.3	227.7±6.5	360.2±11.5	66.2±2.4
Polyphenol fraction	3.2	96.1±2.6	13.4±0.5	396.8±6.1	34.3±0.9	451.8±15.2	22.8±0.7
Aerial parts							
Percolation	34	186.2±5.5	74.2±2.3	628.3±14.2	158.2±6.6	439.5±13.6	646.7±19.2
Ultrasonic extract	21.7	178.0±4.7	56.7±1.4	380±13.6	134.9±3.7	246.7±8.7	97.9±5.7
Polyphenol fraction	7.5	191.2±6.8	82.3±1.9	420.9±13.8	102.0±4.2	69.8±1.4	94.2±4.3

^a IC₅₀ of BHA was 29.3±5.9, vitamin C, 3.7±0.1 and quercetin 3.9±0.2 μg ml⁻¹, respectively.

^b IC₅₀ for vitamin C and quercetin were 16.7±0.6 and 41.2±1.7 μg ml⁻¹, respectively.

^c EDTA used as control (IC₅₀ = 4.2 ± 0.2 μg ml⁻¹).

^d Quercetin used as control (IC₅₀ = 200 ± 13 μg ml⁻¹).

Scavenging of hydrogen peroxide

Briefly, a solution of H₂O₂ (40mM) was prepared in phosphate buffer (pH 7.4). 1.4ml of extracts (12.5-800 μg ml⁻¹) in distilled water was added to 0.6ml of 40mM H₂O₂ solution. After ten minutes, the absorbance of reaction mixture was determined at 230nm against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of H₂O₂ scavenging by the extracts and standard compounds was calculated as follows: % Scavenged (H₂O₂) = [(A₀-A₁)/A₀] × 100 where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the sample of extract and standard (Dehpour *et al.*, 2009).

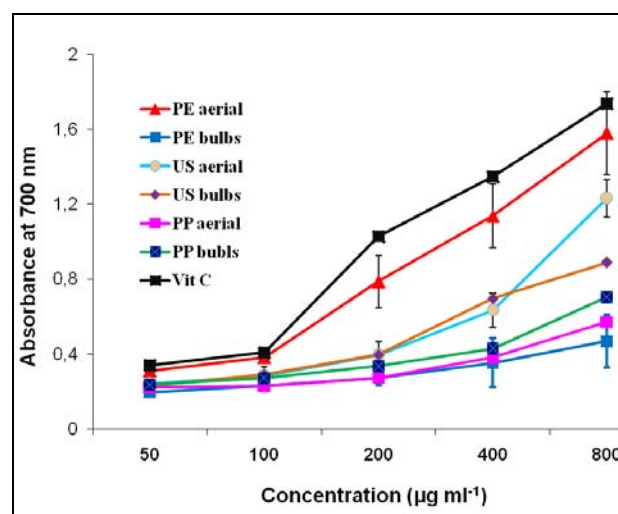


Fig. 1: Reducing power of *Crocus caspius* bulbs and aerial parts; PE (percolation), US (ultrasonic) and PP (polyphenol fraction). Vitamin C used as positive control.

Assay of nitric oxide-scavenging activity

Nitric oxide scavenging activity was measured spectrophotometrically. For the experiment, sodium nitroprusside (10mM) in phosphate-buffered saline (pH 7.4) was mixed with different concentrations of each samples dissolved in water (and methanol as a co-solvent) and left at 25°C for 150min. The same reaction mixture, without extract or essential oil but with an equivalent amount of water, served as control. After the incubation period, 0.5ml of Griess reagent (sulphanilamide (1%) and *N*-1-naphthylethylenediamine dihydrochloride (0.1%) in phosphoric acid (2%) was added. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with *N*-1-naphthylethylene diamine dihydrochloride was measured at 546 nm and the percentage of scavenging activity was measured with reference to standard (quercetin) (Ebrahimzadeh *et al.*, 2010c). The IC₅₀ value is the inhibitory concentration at which nitric radicals were scavenged by 50%.

RESULTS

Total phenolic compounds were reported as GAE by reference to standard curve ($y= 0.0054x+ 0.0628$, $r^2= 0.987$). Aerial parts had higher total phenolic contents than bulbs in all extractions and were in order of: polyphenol (PP) fraction > percolation (PE) extract > ultrasonic (US) extract, respectively. The total flavonoid contents had the same order: PP fraction > PE extract > US extract, respectively by reference to standard curve ($y= 0.0063x$, $r^2= 0.999$) (Table 1). Aerial part extracts showed better activity in scavenging of DPPH free radicals but bulb PP fraction was stronger than aerial parts PP fraction. Figure 1 shows the dose- response curves for the reducing powers of *C. caspius* bulbs and aerial parts. Aerial parts PE extract had shown very potent reducing power which was so better than other extracts ($p< 0.01$). This fraction showed the same activity as vitamin C ($p< 0.05$). Aerial parts PP fraction showed very good Fe^{2+} chelating ability which was better than other fractions (Table 1). Extracts scavenged H_2O_2 dose dependently. IC_{50} were in the same order of inhibiting DPPH radical. Aerial part extracts showed better activity but bulb PP fraction was stronger than aerial parts PP fraction. Extracts also showed good nitric oxide scavenging activity between 12.5 and 1600 $\mu g/ml$. PP fractions showed better than other extracts. Bulb PP fraction with $IC_{50} = 22.8 \pm 0.7 \mu g ml^{-1}$ was the most potent fraction. Quercetin was a better scavenger with $IC_{50}=200 \pm 13 \mu g ml^{-1}$ used as control.

DISCUSSION

Phenolic compounds, including phenolic acid and flavonoids are antioxidant compounds widely prevalent in plants. These compounds also possess various biological activities and are able to reduce the incidence of cardiovascular diseases, cancer and degenerative diseases (Pietta, 2000). Aerial parts had higher total phenolic contents than bulbs in all extractions. The total flavonoid contents had the same order. Extracts showed a concentration-dependent radical scavenging activity by inhibiting DPPH radical. The scavenging activity on DPPH free radicals represents a widespread and accepted method for assessing the antioxidant capacity of an extract. This method relies on the ability to donate hydrogen radicals to DPPH. As a result, there is a color change from violet to yellow, and the monitoring of absorbance decrease at 517 nm. Substances which are able to perform this reaction can be considered as antioxidants and radical scavengers. Aerial part extracts showed better activity but bulb PP fraction was stronger than aerial parts PP fraction. Aerial parts US extract with high levels of phenol and flavonoids were the most potent extract (Table 1). In the assay of reducing power, the presence of reductants (antioxidants) in the samples could result in the reducing of Fe^{3+} to Fe^{2+} ions. Depending on

the amount of antioxidants in an extract, the reduction of Fe continues to take place. This is used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (Ebrahimzadeh, *et al.*, 2010b). Some molecules work by breaking the chain of free radicals and by donating hydrogen atoms, but can, in addition, bind some precursors of peroxide, thus preventing the formation of this radical. Amount of reduction can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance indicates an increase in reductive ability. It was found that the reducing powers of extracts also increased with the increase of their concentrations. Aerial parts PE extract had shown very potent reducing power which was so better than other extracts ($p< 0.01$). This fraction showed the same activity as vitamin C ($p< 0.05$). The transition metal ion, such as iron, possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions. Ferrous ions can initiate lipid peroxidation by the Fenton reaction, as well as accelerating peroxidation (Ebrahimzadeh *et al.*, 2009b). Ferrozine can make complexes with ferrous ions. Reduction of Fe^{2+} concentration leads protection against oxidative damage. In the presence of chelating agents, a complex (colored in reddish-pink) formation is interrupted and as a result, the colour of the complex is decreased. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, improving that it has chelating activity and captures ferrous ion before ferrozine. According to Table 1, the chelating effects of extracts increase with sample concentration from 25 to 800 $\mu g ml^{-1}$. It was reported that chelators are effective as secondary antioxidants because by stabilizing the oxidized form of the metal ion, they reduce redox potential. Aerial parts PP fraction showed very good Fe^{2+} chelating ability which was better than other fractions (Table 1). Scavenging of hydrogen peroxide by extracts may be attributed to their phenolic contents, which can donate electrons to hydrogen peroxide and neutralize it to water. The differences in H_2O_2 scavenging capacities between the extracts may be attributed to the structural features of their active components which determine their electron donating abilities. Extracts scavenged H_2O_2 dose dependently. IC_{50} were in the same order of inhibiting DPPH radical. Aerial part extracts showed better activity but bulb PP fraction was stronger than aerial parts PP fraction. Aerial parts US extract with high levels of phenol and flavonoids were the most potent extract (table 1). Although H_2O_2 itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems (Ebrahimzadeh *et al.*, 2010a). Extracts also showed good nitric oxide scavenging activity between 12.5 and 1600 $\mu g/ml$. The inhibition increased with increasing concentration of the extract. PP fractions showed better than other extracts.

Bulb PP fraction was the most potent fraction. In addition to reactive oxygen species, NO is also implicated for other pathological conditions such as inflammation and cancer. Scavenging activity of nitric oxide may help to stop the chain of reactions initiated by the detrimental excess generation of NO (Ebrahimzadeh *et al.*, 2010a).

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