In-Vitro Antioxidant and Anti-Tyrosinase Activity of Methanol Extracts From *Crocus* Sativus Flowers

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Summary

Methanolic extract of Crocus sativus flowers was prepared, and its antioxidant activity evaluated by total phenolic contents (TPC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity (ABTS) and the reducing power. The results showed that TPC was 86.65 mg/g gallic acid equivalents, while DPPH and ABTS for 1 mg/mL concentration were 92.41 and 86.87, respectively. In the reducing power experiment, the IC50 value was 231.75, about 11 times lower than that of ascorbic acid. The extract showed a significant inhibitory effect on tyrosinase activity of 28.22%.

Keywords: Antioxidant; Tyrosinase inhibition, Crocus sativus flowers, saffron.

1. Introduction

During normal metabolic processes or due to the exogenous factors and agents, reactive oxygen species (ROS) in the forms of superoxide anion radical ($\cdot O^{2-}$), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2) may be generated. Formation of ROS can cause oxidative damage to human cells, leading to various diseases such as cancer, cardiovascular disease, osteoporosis, and degenerative diseases (1). Some compounds known as antioxidants are able to delay or inhibit the initiation or propagation of oxidative chain reaction and thus prevent or repair oxidative damage done to the body's cells by oxygen (2). During the last few decades, some synthetic antioxidants such as butylated hydroxyanisol (BHA) and butylated hydroxy toluene (BHT) have been used or suggested as additives in food industry. However, due to their synthetic origin, these antioxidants may induce carcinogenesis by mutagenicity and toxicity against human enzymes and lipids (3). However, antioxidants derived from natural sources have attracted many interests for use in foods or pharmaceutical preparations. Particularly, much study is focused on natural products including vegetables and wild plant sources (4).

Tyrosinase (monophenol monooxygenase, E:C:1.14. 18.1), also known as polyphenol oxidase (PPO) is a copper containing enzyme. The enzyme is involved in the first two steps of melanin biosynthesis (5). Melani formation is the main cause of enzymatic browning in plants, fruits and human skin. It is known that biosynthesis of melanin leads

to undesirable changes in color, flavor and nutritive values of plant-derived foods and beverages (6). Therefore, tyrosinase inhibitors have become increasingly important in cosmetic, food and pharmaceutical products in relation to hyperpigmentation (7, 8). The tyrosinase inhibitors such as catechins have been identified in some natural sources especially from various parts of certain plants (9).

Crocus is a genus in the iris family comprising about 80 species of perennials growing from corms. Many are cultivated for their flowers appearing in autumn, winter, or spring. Crocuses are native to woodland, scrub and meadows from sea level to alpine tundra in central and southern Europe, North Africa and the Middle East, on the islands of the Aegean, and across Central Asia to western China.

The domesticated saffron crocus (*Crocus sativus*) is an autumn-flowering perennial plant unknown in the wild. Its major growing region is East and South East of Iran and our country is one of the few countries producing saffron. It is often mistaken for the more plentiful common autumn crocus, which is also known as meadow saffron or naked ladies (Colchicum autumnale) and has been the cause of deaths due to mistaken identity. Saffron, this most expensive spice, is the stigma of Crocus sativus flower, or saffron crocus (family Iridaceae) (10, 11). Each of the large blooms of the saffron crocus offers three stigmas inside the flower that must be picked by hand and then dried. The flower itself is discarded after isolation of its valuable stigma.

It has been demonstrated that saffron contains more than 150 carotenoid compounds including safranal, zeaxanthin, lycopene and various α- and β-carotenes, Figure 1 (10). It is also a concentrated source of riboflavin (12). The characteristic yellow color of saffron is from a water-soluble pigment, the carotenoid crocin (10). However, a specific component named picrocrocin is partly responsible for its characteristic pleasant flavor. Saffron possesses a variety of pharmaceutical and medicinal applications. It has been found that safranal together with other carotenoids derived from saffron, could reduce inflammation, prevent liver and spleen enlargement, restrict urinary bladder and kidney infection, prevent menstrual disorders and inhibit catarrhal infections (13-19). A major compound found in saffron, crocetin, could indirectly help to reduce cholesterol levels in the blood and the severity of atherosclerosis, i.e. lower the risk of heart attacks (20, 21). Saffron is, however, highly toxic when ingested in a large amounts by young animals (15).

Considering the various medicinal benefits of saffron in one hand and large volume of waste flowers in Iran, the aim of this study was investigating antioxidant and anti-tyrosinase activity of other parts of flower after separation of stigma. It is worth indicating that up to now, the large volume of used flowers in Ghaenat (East of Iran) are discarded and, therefore, their possible medicinal value could open in new industry perhaps more beneficial than saffron alone.

2. Materials and methods

Fresh Crocus sativus flower wastes (*Camellia sinensis* (L.) were collected from domestic saffron producing places in Ghaenat (Eastern Iran) during autumn 2010. The waste is considered as fresh or a few hours old flowers remained after hand separation of stigma from the original flower. They were immediately transported to our laboratory, washed and drained. 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH), potassium phosphate buffer, hydrogen peroxide, sodium acetate, dimethyl sulfoxide (DMSO), ferric chloride, gallic acid, Folin-Ciocalteu's phenol reagent and ferrous sulphate were purchased from Sigma representative in Iran. All solvents were of reagent grade and obtained from Merck representative in Iran.

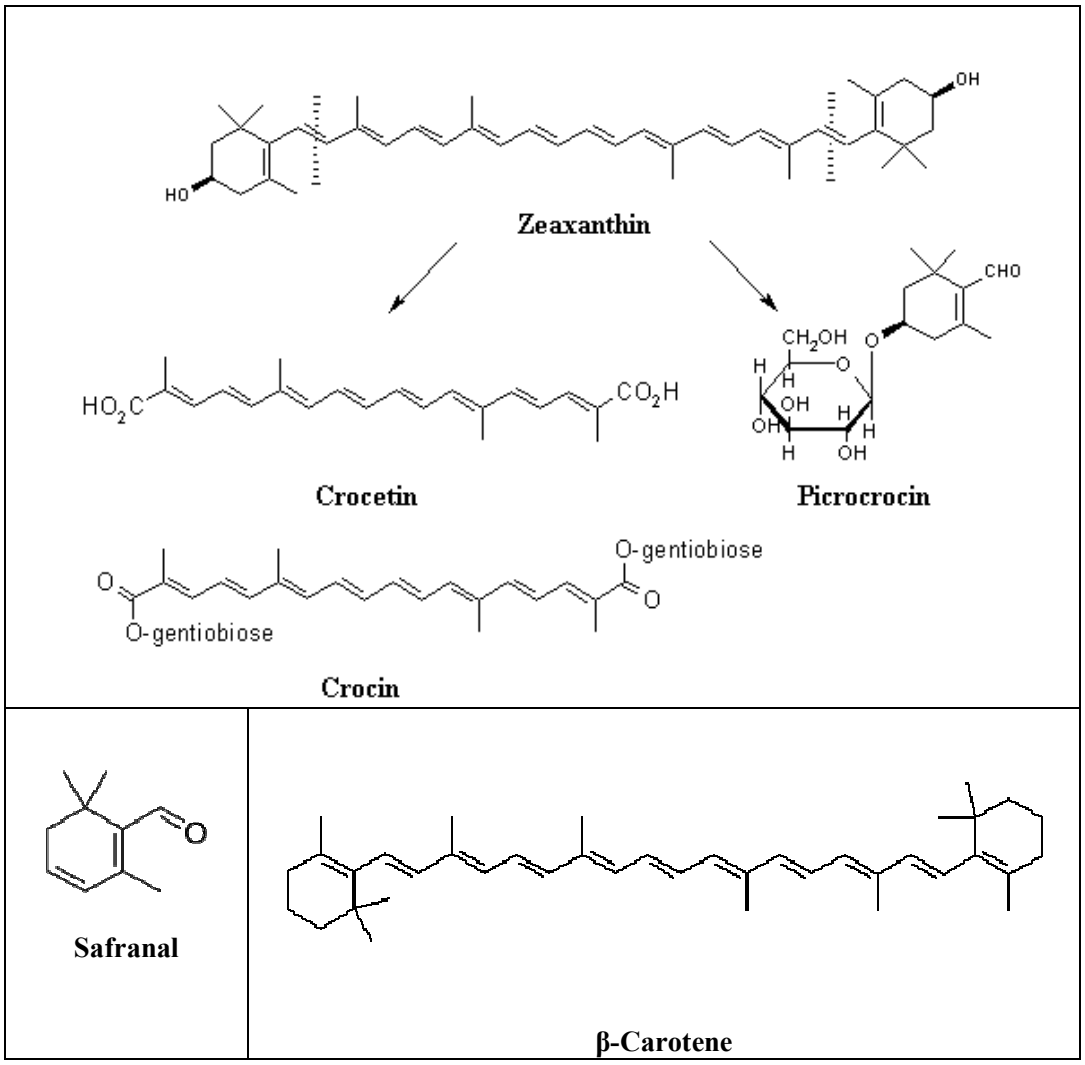

Figure 1. Some carotenoid compounds found in saffron.

2.1. Preparation of methanolic extract from Crocus sativus flower wastes

Each 10 g of fresh Crocus sativus flower was extracted with 200 mL of methanol in a shaking incubator (100 rpm) overnight at room temperature and the extracts were filtered through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan). Solvent were then removed by evaporation in vacuo, to obtain the dried extracts. The dried extracts were then dissolved in DMSO with concentration of 50 mg/mL for the experiment, and diluted with DMSO if necessary.

2.2. Total phenolic content (TPC) by the Folin–Ciocalteu assay

The total phenolic content (TPC) of Crocus sativus flower extract was determined using a modification of the procedures described by Gutfinger (22). Briefly, one milliliter of extract was mixed with 1.0 mL of 2% Na_2CO_3, and the mixture was allowed to stand at room temperature for 3 min. 0.2 mL 50% Folin-Ciocalteu reagent was then added and the reaction was left to complete for 30 min in the dark. The mixture was centrifuged at 14,000 × g for 5 min. The absorbance of supernatant was measured at 650 nm on a single beam UV-visible spectrophotometer (Ultrospec 3000 from Pharmacia Biotech) and TPC was expressed as gallic acid equivalents. It should be mentioned that all of the experiments in this research were carried out in triplicate.

2.3. DPPH radical scavenging assay

A similar method introduced by Lee (23) was used to determine the DPPH radical scavenging activity of Crocus sativus flower extract. In practice, 0.1 mL of extract was mixed with 0.9 mL of 0.041 mM ethanolic DPPH solution. The mixture was then vortexed vigorously and allowed to stand for 10 min in the dark. The absorbance of the sample was measured at 517 nm against a blank and using ascorbic acid as positive control. Radical scavenging activity was calculated using the following relationship:

$$\% \text{ Inhibition} = [(A_B - A_A) / A_B] \times 100$$

Where: A_B-absorption of blank sample (t=0 min); A_A-absorption of extract solution (t=15 min).

2.4. ABTS radical scavenging activity

The determination of ABTS was performed according to procedure described by Muller (24) with slight modifications. In a typical experiment, 0.1 mL of potassium phosphate buffer (0.1 M, pH 5.0), was mixed with 20 µl of hydrogen peroxide (10 mM) and 0.1 mL flower extract. The mixture was then incubated at 37 °C for 5 min. A mixture of 30 µl ABTS (1.25 mM, in 0.05 M phosphate–citrate buffer, pH 5.0) and 30 µl peroxidase (1 unit/mL) were then added and further incubated at 37 °C for another 10 min. The absorbance of mixture (A_A) was measured at 405 nm against a blank (A_B) on a multiplate reader (Tecan Austria) using ascorbic acid as positive control. The ABTS radical scavenging activity was measured from the following relationship:

$$\text{ABTS radical scavenging activity (\%)} = [1-(A_A/ A_B] \times 100$$

2.5. Reducing power (RP) assay

The reducing power of Crocus sativus flower extract was measured by a modification of the method used by Oyaizu (25). 1.0 mL of the extract was mixed with 1.0 mL sodium phosphate buffer (0.2 M, pH 6.6) and 1.0 mL of potassium ferricyanide (10 mg/mL). The mixture was incubated at 50 °C for 20 min followed by addition of 1.0 mL trichloroacetic acid (100 mg/mL) and centrifugation at 14,000 × g for 5 min. The supernatant (1.0 mL) was mixed with 1.0 mL distilled water and 0.1 mL of ferric chloride 0.1%, and the absorbance was measured at 700 nm against a blank.

2.6. Tyrosinase assay and inhibition studies

The biological activity of mushroom tyrosinase was measured spectrophotometrically using kojic acid as a standard tyrosinase inhibitor (26). The enzymatic reaction was initiated by addition of a known amount of the enzyme to substrate solution containing dimethyl formamide (DMF and MBTH). DMF was added to the reaction mixture in order to keep the resulting colored complex in soluble state during the course of investigations. The progress of the reaction was followed by measuring the intensity of the resulting pink color at 505 nm. A typical reaction mixture with a total volume of 1.0 ml contained 100 µl enzyme solution (a), 500 µl substrate solution (b) and 400 µl phosphate buffer (pH 6.8). To investigate the effect of Crocus sativus flower extracts on the activity of enzyme, the phosphate buffer was replaced by 400 µl of extract (3-6 mg of dry flower extract was dissolved in 1.0 mL phosphate buffer, pH 6.8). The 50% inhibition (IC_{50}) of tyrosinase activity was calculated as the concentrations of each sample that inhibited 50% of tyrosinase activity. The resulting data were expressed as a percentage of inhibition of tyrosinase activity.

2.7. Statistical analysis

All measurements were carried out in triplicate, and analysis of variance was conducted according to the procedure of the General Linear Model using SAS software (SAS Institute, NC, USA, 1995). Student–Newman–Keul's multiple-range tests were used to compare the significant differences of the mean value among treatments ($P < 0.05$). *P* values less than 0.05 were considered statistically significant.

3. Result and discussion

3.1. Total phenol content (TPC)

The extraction yield was calculated using the dry weight obtained after extraction of 1000 grams of fresh Crocus sativus flower, followed by solvent evaporation and the result is included in Table 1. Total phenolic compounds in terms of gallic acid equivalent (GAE/g) were found to be 86.65 mg/g. The results are mean values calculated from three experiments performed at similar conditions.

Table 1. Extraction yield and total phenol contents (TPC) of methanol extract of Crocus sativus flower (saffron flower), (P < 0.05), n = 3.

	Saffron flower extract
Fresh flower weight (g)	1000.00
Dry weight (g)	120.22
Dry weight after solvent evaporation (g)	56.14
Total yield (%)	46.77
TPC(mg GAE/g extract)	86.65 ± 0.45

It is known that most polyphenolic compounds are highly reactive scavengers of free radical due to their ability to produce stable free radical intermediates. They, therefore, could act as effective antioxidants. The antioxidant capacity in terms of total phenolic content (TPC) in Crocus sativus flower extract was determined using a linear gallic acid standard curve. The results showed that Crocus sativus flower extract contained a high ($P < 0.05$) TPC when compared to reported results for wisteria floribunda extracts, i.e. 62.23 mg (27) and Magnolia denudate flower extracts, i.e. 0.57 mg (28). On the other hand, the results reported more recently for white and violet Magnolia denudate flower extracts are, however, similar to the values obtained from this study for saffron flower extracts (29). The similarities and differences could be explained in terms of various determinant factors including environment, extraction method, analytical method and the presence or absence of various stresses.

3.2. DPPH radical scavenging activity

DPPH is a stable free radical widely used to evaluate the free radical-scavenging activity (RSA) of various natural products and some synthetic pure compounds. In this part of the research work, DPPH radical scavenging activity of Crocus sativus flower extract was measured using ascorbic acid as a positive control and the results are presented in Table 2. It can be seen that concentration of flower extract (50-1000 µg/mL) plays a direct role on DPPH radical scavenging activity. DPPH radical scavenging activity obtained from our research was higher than the value reported for white Magnolia denudata, and similar to violet Magnolia denudate (29). This can be explained in terms of the presence of strong pigment compounds such as cyanidins. It is known that cyanidins is one of the important compounds that could react as antioxidant (30). Saffron has very pretty flowers with a deep purple color, which can be partly responsible for high antioxidant activity, since it has been reported that the intensity of color in flowers could play an important role in their antioxidant activity due (31). In this research, we found that IC_{50} value for methanolic extracts of saffron flower was about 11 times lower than that of ascorbic acid, which was similar to the IC_{50} of Magnolia. Denudate (29). According to correlation test, it was shown that DPPH radical scavenging activity of Crocus sativus flowers was correlated with its TPC ($r = 0.841$).

Table 2. DPPH radical scavenging activity of methanol extract from Crocus sativus flower compared to ascorbic acid

Concentration (µg/ml)	% DPPH	Ascorbic acid (positive control)
50	19.16 ± 0.22	
100	32.33 ± 0.50	
500	88.65 ± 0.41	
1000	92.41 ± 0.12	
IC_{50}[a]	231.75 ± 1.92	20.99 ± 0.21

[a]IC_{50} (µg/ml), concentration for scavenging 50% of DPPH radicals, ($P < 0.05$), n = 3.

3.3. ABTS radical scavenging activity

The antioxidant ability of the flower extract to scavenge the radical ABTS was also compared to the ascorbic acid. In this case, also the increase in the ability to scavenge ABTS free radicals was obtained by increasing the concentration increased of flower extract (Table 3). It was observed that at almost any concentrations, ABTS was significantly ($P < 0.05$) higher than the value reported by other researchers for Magnolia. Denudate (28-30).

Table 3. ABTS radical scavenging activity of methanolic extracts of Crocus sativus flowers.

Concentration (µg/ml)	ABTS (%)	Ascorbic acid (positive control)
50	12.81 ± 2.34	
100	32.55 ± 4.77	
500	68.77 ± 1.32	
1000	86.87 ± 0.66	
IC_{50}[a]	202.21 ± 14.37	17.28 ± 0.60

[a] IC_{50} (µg/ml), concentration for scavenging 50% of ABTS radicals. $P < 0.05$, n = 3.

3.4. Reducing power (RP) assay

Reducing power of methanolic extract from Crocus sativus flower is compared to that of ascorbic acid in Table 4. The most important result obtained from this study was that the reducing power together with other factors introducing antioxidant activity was significantly ($P < 0.05$) higher than that of other flower extracts reported in literature. We found that IC50 was about 11 times lower than that of ascorbic acid. It has been found that reducing power of methanolic extract from Cherry (Prunus serrulata var. spontanea) blossoms is higher than Crocus sativus flower obtained from our study (32). We also found that the value of reducing power had a linear correlation with the total phenol content (compare Table 1 with 4). This conclusion is in agreement with what was found in (33).

3.5. Tyrosinase inhibitory activity

The inhibitory effect of Crocus sativus flower extract and ascorbic acid (positive control) on biological activity of tyrosinase are presented in Table 5. It can be seen that tyrosinase inhibitory activity of Crocus sativus flower increased with increasing concentration, i.e. from 50 to 1000 µg/mL. It was found that inhibitory effect of Crocus sativus flower extract on tyrosinase activity was 10.78 at concentration of 50 µg/mL and the value reached up to 28.22 at 1000 µg/mL. It was also observed that the extracts exhibited IC_{50} values of about 40 times lower than that of ascorbic acid. These observations are in agreement with the results obtained for a variety of seashore plant species (34). They have reported that the strong antioxidant activity of seashore plant species have also strong anti-tyrosinase ability. Although the tyrosinase inhibitory activity of methanolic extracts was significantly ($P < 0.05$) lower than ascorbic acid, but it is suggested that using different extraction solvents may lead to increase in the anti-tyrosinase activity as well as antioxidant power.

Table 4. Reducing power of methanol extracts from Saffron flower extract.

Concentration (µg/mL)	Reducing power	Ascorbic acid (positive control)
50	0.248 ± 0.003	
100	0.393 ± 0.003	
500	1.122 ± 0.004	
1000	1.178 ± 0.002	
IC_{50}[a]	193.91 ± 1.22	15.69 ± 0.08

[a] IC_{50} (µg/ml), concentration for increasing 0.500 value in optical density. $P < 0.05$, n = 3.

Table 5. Tyrosinase inhibitory activity of methanol extracts from Crocus sativus (saffron) flower.

Concentration (µg/mL)	%Tyrosinase inhibition by Crocus sativus extract	%Tyrosinase inhibition by ascorbic acid
50	10.78 ± 0.36	37.89± 0.45
100	16.17 ± 0.62	63.76± 0.58
500	15.21 ± 0.43	76.36± 0.48
1000	28.22 ± 0.51	98.24± 0.65
IC50[a]	9132.55 ± 278.72	229.68 ± 1.06

[a]IC50 (µg/ml), concentration for inhibitory 50% of Tyrosinase. ($P < 0.05$), n = 3.

4. Conclusions

The results obtained from this study showed that methanolic extracts of remaining flower after separation of stigma (saffron) contain a wide variety of antioxidants, especially phenolic compounds that, if isolated properly, could be used effectively in medicinal, pharmaceutical and food preparations. It was also concluded from our results that a good correlation existed between total phenolic content and antioxidant activities of the extracts. Methanolic extracts from these flowers exhibited comparably high inhibitory effect on tyrosinase activity as compared to literature stated similar extracts representing about 30% of the effect of positive control, ascorbic acid. It is, therefore, predicted that the Crocus sativus flower extracts, traditionally discarded after preparation of saffron stigma, have potential use as ingredient in cosmetic product due to their anti-tyrosinase activity. On the other hand, the results obtained from antioxidant activity in this work demonstrated that Crocus sativus flowers could be considered as sources of natural antioxidant in food industry.

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