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Research Paper

Evaluation of three methods for the extraction of antioxidants from Cucumis melo L. fruit and leaves

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Introduction

Plants and plant-derived products contain a wide range of phytochemicals, such as antioxidants, which are thought to have a protective role against risk of oxidative stress related diseases such as cancer and cardiovascular diseases (Garcia-Alonso et al., 2004). Natural products have been the basis to treat some human diseases. There is emerging interest in the use of naturally occurring antioxidants for the management of a number of pathophysiological conditions which involve free radical damage such as cancer and diabetes (Kaur and Kapoor, 2002). Cucurbitaceae family consists of about 119 genera with 825 species including the genus Cucumis comprising increasing numbers of species to 34 (Chen et al., 1997). Of them, a commercial vegetable crop, melon (*Cucumis melo* L.), is an economically important species (Kirkbride, 1993). Melon is a temperate and warm season crop with vegetable and ornamental use. *C. melo* also called as musk melon. It grows in sandy areas and also near river banks. Within Cucurbitaceae, genus *Cucumis* are considered the most important ones under climatic conditions of the middle Europe (Křistkova et al., 2003). The origin of melon was in Africa (Kerge and Grum, 2000) but the distribution of wild and cultivated melon types is worldwide currently (Pitrat, 1991). A high level of molecular and morphological variability in leaf plant and fruit characters has been described within these species (Monforte et al., 2003). Melons were divided into six groups namely; Cantaloupe, Inodorus, Flexuosus, Conomon, Chito, Dudaim, and Momordica (Munger and Robinson, 1991). In Iran, the Cantaloupensis and Inodorus groups are most important for commercial production (Nastari Nasrabadi et al., 2012). Melon is among the 20 most important vegetable crops worldwide (FAOSTAT, 2008). Earlier studies on the cucurbitaceae family showed that *Cucumis melo* L. pulp extract possesses high antioxidant and anti-inflammatory properties (Vouldoukis et al., 2004). Antioxidant properties of seeds extract on streptozotocin induced diabetic rats has been reported by Sathishsekar and Subramanian (2005). The active principles in these vegetal extracts are principally water soluble or lipophilic antioxidant molecules. Mops of these plant extracts contained various amounts of vitamin E, C, carotenes, triterpenoids and other flavonoids (Aruoma, 2003) and were used as potential antioxidant prophylactic agents for both health and disease management (Peng et al., 2000). Fruit has been traditionally used for the diuretic, antihelmentic and cooling effect. It is a good source for appetite, weight loss, urinary tract infections, constipation, acidity, and ulcers (Desai, 2004). Melons add flavor, aroma, color and variety to the American diet. Melon fruit are not important dietary sources of calories (carbohydrates, fats) or protein. Antioxidant properties of *C. melo* seed has been reported (Gill et al., 2010).

The development of methods to extract bioactive compounds from plant materials, particularly by the pharmaceutical industry, has led to an increased need for ideal extraction methods that can obtain the maximum of the bioactive constituents in the shortest processing time at a low cost. In this study, the efficiencies of three methods used to extract antioxidants were evaluated. In the recent years, in our laboratories, some of the widely used Iranian medicinal plants used in folk medicine have been selected for the investigation of their chemical constituents, pharmacological and biological activities in an attempt to establish a scientific basis for their ethno medical uses (Ebrahimzadeh et al., 2006; 2010a; Mahmoudi et al., 2009; Nabavi et al., 2010). In continuation of our research program, in order to scientifically evaluation of ethnomedical uses of medicinal plants, ultrasonically assisted extraction, soxhlet extraction and maceration method were used for extraction of *Cucumis melo* L. fruit and leaves. The antioxidant capacities of the extracts were measured by 1,1-diphenyl picrylhydrazyl (DPPH) and nitric oxide radical scavenging activities, iron chelatory capacity and reducing power. The total phenolic and flavonoid contents of the extracts were determined using the Folin–Ciocalteu and aluminum chloride method, respectively. A possible relationship between total phenol and flavonoids and pharmacological activities was considered.

Materials and methods

Plant materials and preparation of freeze-dried extract

Cucumis melo L. fruit and leaves were collected, in June 2012 from Tonekabon, Iran. The sample was authenticated by Dr. B. Eslami and the voucher specimen was deposited (No. 1143) have been deposited in the Sari School of Pharmacy herbarium. Plant materials were dried under dark conditions at room temperature. The dry material was coarsely ground, obtaining 2-3 mm particles and then extracted by methanol for 24 h at room temperature. The extracts were then separated from the sample residues by filtration through Whatman No.1 filter paper and repeated three times. The resulting extracts were concentrated over a rotary vacuum at 35-40 °C until a crude solid extracts were obtained which then were freeze-dried for complete solvents removal.

Soxhlet assisted extraction

The powders of samples were extracted exhaustively in a Soxhlet extractor with methanol for 24 hours. The extracts were then concentrated in a rotary evaporator until the solvent was completely removed. The methanol extracts was kept in a well-closed container in refrigerator until use.

Ultrasonically assisted extraction

Samples were extracted with methanol in an ultrasonic cleaning bath by indirect sonication at a frequency of 100 kHz and a temperature of 25 ± 3°C for 1 h to yield ultrasonic extracts. The extracts were then separated from the samples residue by filtration. The resultant extracts were concentrated in a rotary evaporator until a crude solid extracts were obtained which were freeze-dried for complete solvent removal and used as ultrasonic extracts (Rabiei *et al.,* 2012).

Determination of total phenolic compounds and flavonoid contents

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Ebrahimzadeh et al., 2008). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g l^{-1} sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml^{-1} solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (GAE, mg g^{-1} of dry mass), which is a common reference compound. The total flavonoid content was measured by a colorimetric aluminum chloride method (Ebrahimzadeh et al., 2008). Briefly, 0.5 ml solution of extract in methanol were mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415. Total flavonoid contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml^{-1} in methanol.

DPPH radical-scavenging activity

Spectrophotometric analyses were recorded on double beam Perkins Elmer UV/Visible-spectrophotometer to determine the DPPH free radical scavenging ability (Ebrahimzadeh et al., 2010a). Different concentrations of extract were added, at an equal volume to methanolic solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Iron chelating activity

The ability of extract to chelate ferrous ions was estimated by our recently published paper (Ebrahimzadeh et al., 2008). Briefly, different concentrations of extract were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), and the mixture was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was absorbance of mixture containing extract or standard. EDTA was used as a standard.

Assay of nitric oxide-scavenging activity

For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extracts but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (Dehpour et al., 2009).

Reducing power determination

The reducing power of extract was determined according to our recently published paper (Nabavi et al., 2010). 2.5 ml of extract (25-800 µg/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The IC_{50} values were calculated from linear regression analysis.

Results and Discussion

Plants have been used traditionally for the treatment and prophylaxis of different disorders. This protection has been attributed to their antioxidant components such as polyphenols (Prior, 2003). Polyphenols are important components in fruit tissues. These compounds are thought to be instrumental in combating oxidative stress. They can prevent some oxidation-related diseases such as atherosclerosis, cardiovascular and neurodegenerative diseases and cancer (Sun et al., 2009). Polyphenols are important components in fruit tissues. Phenolic compounds are a class of antioxidant compounds which act as free radical terminators (Shahidi and Wanasundara, 1992). Total phenol compounds were measured using Folin Ciocalteu method, are reported as GAE by reference to standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The plant generally had high total phenolic contents. The total phenolic content ranged from 71.2 to 111.8 mg GAE/g of extract. The Folin-Ciocalteu phenol reagent assay is used widely for a crude estimation of the amount of phenolic compounds present in an extract. This method is based on the reducing power of the phenolic hydroxyl groups, which react with the Folin-Ciocalteu phenol reagent to form chromogens that can be detected spectrophotometrically at 760 nm. In general, soxhlet extract of leaves had higher phenolic content than other extracts. In leaves, total phenolic contents were in order of: Soxhlet extract > ultrasonic extract > maceration method, respectively. This plant was a good source of phenols and contains very high amount of total phenolics.

Flavonoids, which have the basic skeleton of diphenylpropanes (C_6 - C_3 - C_6) with different oxidations of the central pyran ring, are widely distributed in the plant kingdom and constitute about half of the 8,000 or so recognized phenols (Heim et al.,

2002). Flavonoids are the molecules responsible for the color of fruit and flowers. As the products of secondary metabolism in plants, they are of interest to the pharmaceutical and food industries because of their reported antioxidant activity (Paniwnyk et al., 2001). Using this method, flavonoids with some specific chemical structure can react with Al^{3+} and form a red complex, which gives a maximum absorption at 510 nm. We therefore can only roughly estimate the amount of flavonoids present in an extract by using the spectrophotometric analysis method. Flavonoids form a ubiquitous group of polyphenolic substances typically produced by plants. Flavonoids are of great interest for their bioactivities, which are basically related to their anti-oxidative properties (Cote et al., 2010). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. Flavonoids may slow the pathogenesis of atherosclerosis and cardiovascular 60 diseases by their ROS scavenging effects. The mechanisms of action of flavonoids are through scavenging or chelating process (Cook et al., 1996). Epidemiological evidence also suggests an inverse relationship between intake of dietary flavonoids and risk of cardiovascular disease (Hertog et al., 1997). The total flavonoid content ranged from 13.4 to 59.5 mg QE/g of extract. Soxhlet extract of leaves had higher flavonoid contents than other extracts, too.

The hydrogen atoms, or the electron donation ability of the extracts, were measured from the bleaching of purple-colored methanol solution of DPPH. As a very stable organic free radical with a deep violet color, DPPH gives maximum absorption at the range of 515 to 528 nm. Generally, antioxidants will react with DPPH, a nitrogen-centered radical converted to 1,1-diphenyl-2-picryl hydrazine, due to its hydrogen-donating ability, at a very rapid rate. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. Substances which are able to perform this reaction can be considered as antioxidants and radical scavengers. This method has been used widely to evaluate the radical scavenging ability of antioxidants from different plants due to its advantage of short time and sensibility (Koleva et al., 2002). The capacity of extract to scavenge DPPH was measured and the results are shown in Table 1. Extracts showed a concentration-dependent antiradical activity by inhibiting DPPH radical. This method is based on the reduction of DPPH solution in the presence of a hydrogen or electron donating antioxidant. Substances which are able to perform this reaction can be considered as antioxidants and radical scavengers. Maceration extract of leaves showed the best activity (IC_{50} = 115.0 ± 3.3 µg ml^{-1}) followed by Soxhlet extract of leaves with IC_{50} = 445.3 ± 16.4 µgml^{-1}. Soxhlet extract of both leaves and fruit had high amount of flavonoid contents (Table 1). IC_{50} of standard compound BHA was 53.8 ± 3.7, vitamin C, 3.7 ± 0.1 and quercetin 3.9 ± 0.2 µg ml^{-1}, respectively. So the DPPH scavenging ability of the extracts may be attributed to its hydrogen donating ability that probably shows the role of phenols and flavonoids existing in the extract.

Table 1. Phenol and flavonoids contents and antioxidant activities of *Cucumis melo* L. fruit and leaves.

Part (yield)	Total phenolic contents (GAE mg g^{-1})	Total flavonoid contents (QE mg g^{-1})	DPPH radical scavenging, IC_{50} (µg ml^{-1})[a]	NO scavenging activity, IC_{50} (µg ml^{-1})[b]	Fe^{2+} chelating ability, IC_{50} (µg ml^{-1})[c]
Fruit					
Ultra (20.5)	88.6 ± 1.9	17.0 ± 0.9	780.1 ± 22.5	127.9 ± 3.6	92.0 ± 3.5
Mac (21.9)	77.6 ± 2.4	13.4 ± 0.7	975.5 ± 37.6	251.0 ± 11.0	149.7 ± 3.9
Sox (30.8)	80.9 ± 2.3	58.9 ± 1.5	643.8 ± 19.8	420.7 ± 13.2	57.0 ± 1.8
Leaves					
Ultra (13.4)	72.4 ± 2.8	19.5 ± 0.8	582.7 ± 14.1	376.7 ± 14.9	387.3 ± 11.9
Mac (15.6)	71.2 ± 1.2	18.7 ± 0.6	115.0 ± 3.3	492.8 ± 19.5	360.2 ± 13.3
Sox (16.6)	111.8 ± 2.6	59.5 ± 0.9	445.3 ± 16.4	708.7 ± 13.7	74.2 ± 2.1

Ultra: Ultrasonic; Mac: Maceration; Sox: Soxhlet.
[a] IC_{50} of BHA was 53.8 ± 3.7, vitamin C, 3.7 ± 0.1 and quercetin 3.9 ± 0.2 µg ml^{-1}, respectively.
[b] IC_{50} for quercetin was 155.0 ± 6.4 µg ml^{-1}.
[c] EDTA used as control (IC_{50} = 17.4 ± 0.4 µg ml^{-1}).

Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydro peroxide decomposition reactions via Fenton chemistry. Therefore, minimizing Fe^{2+} concentration affords protection against oxidative damage. Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in humans and thereby improves quality of life and overall survival in some diseases such as Thalassemia (Van Acker et al., 1996). Clinically useful iron chelators have some adverse effects which remain urgent need to identify other chelators with an acceptable degree of tolerability. Therefore, much research has focused on natural product (Ebrahimzadeh et al., 2008). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. Soxhlet extract of fruit showed the best activity (IC_{50} = 57.0 ± 1.8 µg ml^{-1}) followed by Soxhlet extract of leaves with IC_{50} = 74.2 ± 2.1

µgml^{-1}. Soxhlet extract of both leaves and fruit had the highest amount of phenol and flavonoid contents (Table 1). EDTA showed very strong activity (IC_{50} = 17.4 ± 0.4 µg ml^{-1}).

NO transmits signals from vascular endothelial cells to vascular smooth muscle cells and plays an important role in vital physiologic functions many systems. It participates in pathways underlying a large group of disorders such as stroke, muscle diseases, primary headaches, inflammation and neurodegenerative disorders such as Alzheimer disease (Ebrahimzadeh et al., 2010). In the nervous system, NO works as an atypical neural modulator that is involved in neurotransmitter release, learning and memory (Aliev et al., 2009). The scavenging of NO is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of nitrite ions. In such situations, the use of herbal remediation a NO scavenger may prove useful. Extracts exhibited good nitric oxide-scavenging activity. The fruit ultrasonic extract showed the best NO radical scavenging (IC_{50}=127.9 ± 3.6 µg ml^{-1}). It was more potent than quercetin which used as standard (IC_{50}=155.0 ± 6.4 µg ml^{-1}) ($p< 0.01$). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation and other pathological conditions (Moncada et al., 1991).

Reducing power has been used as an antioxidant capability indicator of medicinal Herbs. Fe^{3+} reduction is an important mechanism of phenolic antioxidant action and often used as an indicator of electron donating activity. In this assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Ebrahimzadeh et al., 2010). Increasing absorbance at 700 nm indicates an increase in reductive ability. The greater the intensity of the color, the higher is the antioxidant activity of the sample. Figure 1 shows the dose- response curves for the reducing powers of *Cucumis melo* L. fruit and leaves. It was found that the reducing powers of extracts also increased with the increase of their concentrations. Fruit ultrasonic and maceration extracts had shown very potent reducing power which was comparable to that of vitamin C which used as standard ($p> 0.05$). High phenolic content in these extracts may lead to higher reducing power.

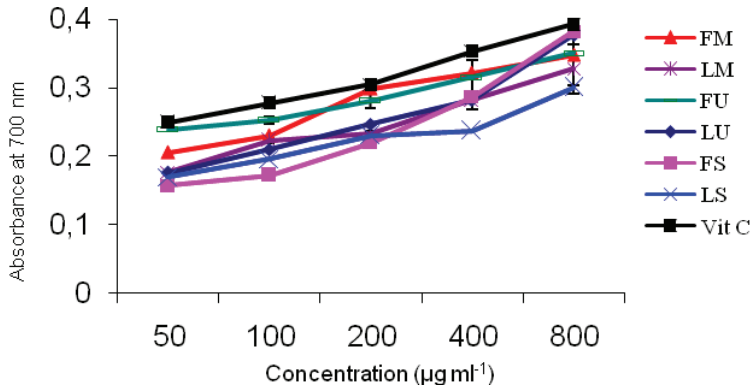

Figure 1. Reducing power of *Cucumis melo* L. fruit and leaves. Vitamin C used as positive control.

Conclusions

The extraction efficiencies of three methods for the extraction of antioxidants from *Cucumis melo* L. fruit and leaves were evaluated. The results obtained indicated that these extraction methods could effectively extract antioxidants from this plant. These extraction methods can be applied to the analysis and purification of antioxidants in plants. This study also successfully identified *Cucumis melo* L. fruit and leaves with very high antioxidant capacities, which are potentially rich sources of natural antioxidants. Further investigation of individual compounds, with their *in vivo* antioxidant activities is needed.

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