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Full Length Research Paper

In vitro antioxidant activity, total phenolics and flavonoids from celery (*Apium graveolens)* leaves

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Apium graveolens **has several beneficial properties including antioxidant activity. The leaves of *A. graveolens* was extracted with methanol and partitioned with water, ethyl acetate and butanol. The phenolic content of the extracts was determined by Folin-Coicalteu method and antioxidant activity was assayed through some *in vitro* models such as antioxidant capacity by radical scavenging activity using α, α-diphenyl-β-picrylhydrazyl (DPPH), β-carotene-linoleate, reducing power, metal chelating effects and phosphomolybdenum method. The phenolic content of the extracts were expressed as gallic acid equivalents and was found to be highest in methanol (51.09 mg/g) followed by water extract (46.40 mg/g), ethyl acetate (22.70 mg/g) and butanol extract (19.43 mg/g). At 250 µg/ml concentration, the antioxidant and free radical scavenging activities of the extracts assayed through DPPH and reducing power were found to be highest with methanol extract followed by water, ethyl acetate and butanol extract. The antioxidant activity of butylated hydroxyl toluene (BHT) was higher than the extracts at each concentration points. The results indicated that the extent of antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract and the celery leaf vegetable being rich in phenolics may provide a good source of antioxidants.**

Key words: *Apium graveolens*, antioxidant activity, α-diphenyl-β-picrylhydrazyl (DPPH), flavonoid content, reducing power, total phenolic content.

INTRODUCTION

Apium graveolens L. (Apiaceae), celery, is a hepaxanthic herb grown as a biennial and under certain conditions, as an annual. Celery is a native of Eurasia and is grown mainly in coastal regions. Celery is widely cultivated in the temperate zones as an important garden crop and the bleached leaf stalks are relished as a popular vegetable. Celery seeds are used in India to treat bronchitis, asthma, liver, and spleen diseases (Satyavati and Raina, 1976). *A. graveolens* is one of the ingredients in 8 of the 33 Indian polyherbal formulations with reputed life-protecting activity (Handa et al., 1986). Several components from celery seeds were also reported for their anticarcinogenic activity (Zheng et al., 1993). Celery seeds possess a characteristic aroma and pungent taste and are used as a condiment in the flavoring of food products. The characteristic odor of celery essential oil is due to a series of phthalide derivatives. Celery is also used as an effective remedy for various ailments such as bronchitis, liver and spleen disease, arthritic pain and this natural holistic approach to health is becoming more and more popular now a days (Kolarovic et al., 2010). The major bioactive compounds in the celery include a class of phenolic compounds called furanocoumarins. The main linear furanocoumarins in celery include the three phototoxic furanocoumarins, Psoralen, xanthotoxin and bergapten (Berenbaum, 1987).

Vegetables and fruits are considered to be good sources of functional ingredients. Leafy vegetables apart from being a good source of minerals also contain antioxidant vitamins and pigments. Leafy vegetables are also known for their therapeutic value. Many studies have shown that antioxidants, present in plants at high levels, are the compounds responsible for these functionalities (Sugimura, 2002). Antioxidants or molecules with radical scavenging capacity are thought to exert a potential

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protective effect against free radical damage. These biomolecules contribute to prevention of coronary and vascular diseases and of tumor formation by inhibiting oxidative reactions (Kris-Etherton et al., 2002). This oxidative damage is the result of free radical action on, for instance, lipids or DNA (Vinson et al., 1998).

However, the commonly used synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) are restricted by legislative rules because they are suspected to have some toxic effects and as possible carcinogens (Imaida et al., 1983). Therefore, there has been a considerable interest by the industry and a growing trend in consumer preferences for natural antioxidants over synthetic compounds and elimination of synthetic antioxidants in food applications has given more impetus to explore natural source of antioxidants. Thus, antioxidants are of interest to both food scientists and health professionals and there has been a convergence of interest among researchers in these fields as the role of antioxidants in the diet and their impact on human health has come under attention. Recently, Kolarovic et al., (2010) has reported the antioxidant activities of celery and parsley juices in rats treated with doxorubicin, but no systematic efforts has been made to study in detail about the antioxidant activity of the celery grown in the Korean region.

The aim of our study was to investigate the antioxidant activity of extracts of different polarity from leaves of *A. graveolens* through non-enzymatic methods. According to the recommendations, the antioxidant effects in more than three different bioassays were studied, besides determination of total phenolics and flavonoids. To the best of our knowledge, this is the first report on the antioxidant activity of the leaves from *A. graveolens* through non-enzymatic approach.

MATERIALS AND METHODS

Plant material and sample preparation

The *A. graveolens* leaves were procured from local market in October 2010 at Gwangjin-gu, Seoul, South Korea. A voucher specimen is deposited in the Department of Applied Life Science, Konkuk University, Seoul, South Korea, and identified by Dr. Ill-Min Chung. The leaves were washed thoroughly in tap water to remove adhering mud particles, rinsed in distilled water, drained, and dried in a hot air oven at 50 ± 2℃. The dried leaves were finely powdered. The dried powder was extracted with 5 × 2 L methanol for 24 h. After removal of the solvent in vacuo, the crude extract was suspended in 0.5 L distilled water and extracted with 0.2 L portions of ethyl acetate and butanol until the extracts were nearly colorless. Solvents were removed in vacuo, and extracts were obtained, respectively.

Determination of total phenolic contents

The total phenolic content was determined by the Folin-Coicalteu (FC) method (Singleton and Rossi, 1965). Distilled water (3.16 ml) was mixed with a DMSO solution of the test compound (40 µl). Then, 200 µl of FC reagent was added. After 5 min, 600 µl of 20% sodium carbonate solution was added and the solutions were mixed again. The solutions were left at room temperature for 2 h. Then the absorption of the developed blue color was determined at 765 nm, using a Macasys Optizen 2120UV plus UV-spectrophotometer (Mecasys, Korea). The concentration of the total phenolic content was determined as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The estimation of phenolic compounds in the fractions was carried out in triplicate and the results were averaged.

Determination of total flavonoid contents

Total flavonoid content of celery leaves were determined by using the aluminium chloride colorimetric method as described (Willet, 2002), with some modifications. Extracts (0.5 ml), 10% aluminium chloride (0.1 ml), 1 M potassium acetate (0.1 ml) and distilled water (4.3 ml) were mixed. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm using a Macasys Optizen 2120UV plus UV-spectrophotometer (Mecasys, Korea). Quercetin was used to make the calibration curve. The estimation of total flavonoids in the extracts was carried out in triplicate and the results were averaged.

α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity

Radical scavenging activity of the celery extracts was determined as described by Katerere and Eloff (2005), with some modification. Different concentrations (50 to 250 µg) of the extracts and BHT were taken in different test tubes. The volume was adjusted to 3.0 ml by adding methanol. One milliliter of 0.1 mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 37℃ for 20 min. The control was prepared as above without any extract and methanol was used for the baseline correction. The changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

Percentage (%) radical scavenging activity = (Control OD – Sample OD/Control OD) × 100.

Antioxidant activity using β- carotene-linoleate model system

The antioxidant activity of the celery extracts were evaluated using β-carotene-linoleate model system as described by Jayaprakasha et al. (2001) with some modification. 0.1 mg of β-carotene in 0.2 ml of chloroform, 10 mg of linoleic acid and 100 mg of Tween-20 (polyoxyethylene sorbitan monopalitate) were mixed. Chloroform was removed at 40℃ under vacuum and the resulting mix ture was diluted with 10 ml of water and was mixed well. To this emulsion, 2.0 ml of oxygenated water was added. Four milliliter aliquots of the emulsion were pipetted into different test tubes containing 0.2 ml of extracts and BHT (50, 100, 150, 200 and 250 µg) in methanol. BHT was used for comparative purposes. A control containing 0.2 ml of methanol and 4 ml of the above emulsion was prepared. The tubes were placed at 50℃ in a water bath and the absorban ce at 470 nm was taken at zero time (t = 0). Measurement of absorbance was continued till the color of β-carotene disappeared in the control tubes (t = 60 min) at an interval of 15 min. A mixture prepared as mentioned in the foregoing without β-carotene served as blank. All determinations were carried out in triplicate.

Table 1. Total phenolic (expressed as gallic acid equivalents) and flavonoid content (expressed as mg quercetin/g) from leaves extracts of *A. graveolens.*

Extract	Total phenolic content (mg/g)	Total flavonoid content (mg/g)
Ethyl acetate	22.70 ± 1.56	4.08 ± 0.31
Methanol	51.09 ± 1.44	2.12 ± 0.08
Butanol	19.43 ± 0.88	4.80 ± 0.03
Water	46.40 ± 0.31	0.77 ± 0.01

Values are means of triplicates ± SD.

The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula:

$AA = 100\ [1-(A_0 - A_t)/ (A^0_0 - A^0_t)]$

Where A_0 and A^0_0 are the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A^0_t are the absorbance measured in the test sample and control respectively, after incubation for 60 min. The results were expressed in percentage basis of preventing bleaching of β-carotene.

Assay of reductive potential

The reductive potential of the celery extracts was determined according to the method of Dorman and Hiltunen (2004). The reaction mixture containing varying concentrations of the extracts and standard (50 to 250 µg/ml) in 1 ml of distilled water, phosphate buffer (2.5 ml, 0.2 M, 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, which was then centrifuged at 1000 rpm for 10 min. The upper layer of the 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 in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased reductive potential. All analysis were run in triplicate and averaged.

Chelating effects on ferrous ions

The chelating effect was determined according to the method of Dinis et al. (1994). Briefly, 2 ml of various concentrations (50, 100, 150, 200 and 250 µg/ml) of the extracts in methanol was 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). Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The inhibition percentage of ferrozine-Fe^{2+} complex formation was calculated by using the formula given below:

$\text{Metal Chelating effect (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$

Where $A_{control}$ is the absorbance of control (The control contains $FeCl_2$ and ferrozine complex formation molecules) and A_{sample} is the absorbance of the test compound. EDTA was used as a standard.

Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of the celery leaves extracts was evaluated by the method of Prieto et al. (1999). An aliquot of 0.1 ml of sample solution (1 mg/ml) was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. For samples of unknown composition, water soluble antioxidant capacity was expressed as equivalents of α- tocopherol (mg/g of extract).

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

The total phenolic content of the extracts from leaves of *A. graveolens* was determined by Folin-Coicalteu method and the results are expressed as equivalents of gallic acid (Table 1). Among the four extracts, methanol extract had the highest (51.09 mg/g) amount of phenolic compounds followed by water (46.40 mg/g), ethyl acetate (22.70 mg/g) and butanol extract (19.43 mg/g). The result of the total flavonoid contents of the extracts from *A. graveolens* leaves is presented in Table 1. The total flavonoid content varied from 0.77 to 4.80 mg quercetin/g weight. The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to gallic acid. It has been observed that the phenol antioxidant index, a combined measure of the quality and quantity of antioxidants in vegetables (Elliot, 1999). The phenolic compounds are the dominant antioxidants that exhibit scavenging efficiency on free radicals and reactive oxygen species are numerous and widely distributed in the plant kingdom (Prior and Cao, 2000).

DPPH radical scavenging activity

The free radical-scavenging activity of the celery extracts was tested through DPPH method (Katerere and Eloff, 2005) and the results were compared with BHT. DPPH is

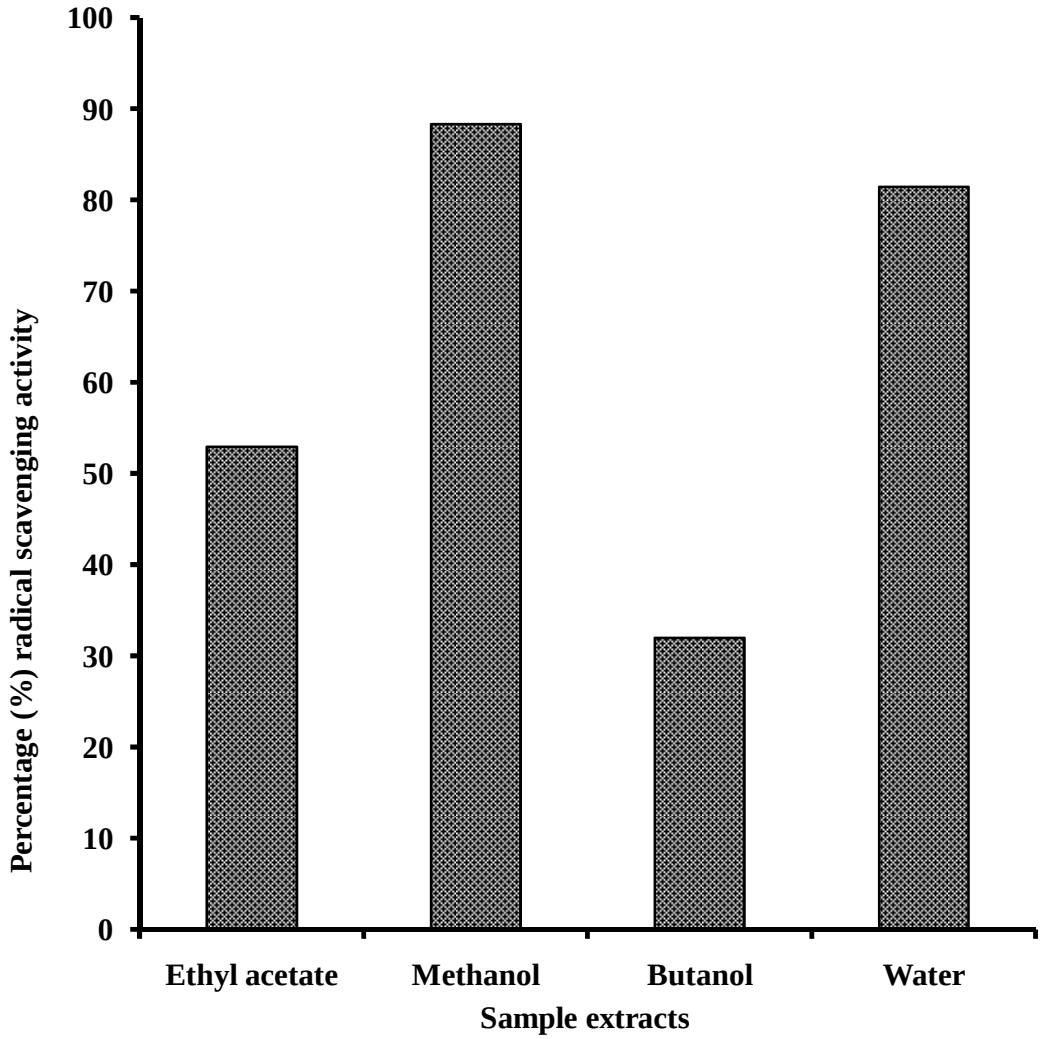

Figure 1. Free radical-scavenging activity of the extracts from celery leaves at 250 µg/ml concentration by DPPH method. Each sample was assayed in triplicate for each concentration. Experimental results are means ± SD of three parallel measurements.

usually used as a substrate to evaluate antioxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extracts were able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. The IC_{50} values of the extracts were, methanol (88.73 µg/ml), water (95.11 µg/ml), ethyl acetate (236.20 µg/ml) and butanol (391.23 µg/ml), respectively. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., ρ-phenylene diamine, ρ-aminophenol), reduce and decolorize 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability (Blois, 1958).

The positive correlation between polyphenolic content of the extracts and its antioxidant activity is well documented (Huang and Mau, 2006). Therefore, the content of total phenolic compounds in the extracts might explain their high antioxidant activities. In this study, the extracts exhibited a concentration-dependent antiradical activity by inhibiting DPPH radical. Of the different extracts, methanol extract exhibited the highest antioxidant activity of 88.31% at 250 µg/ml concentration, followed by water (81.44%), ethyl acetate (52.92%) and butanol extract (31.95%), respectively at the same concentration (Figure 1).

One of the possible mechanisms is polyphenolic-associated compounds (formation of non-extractable

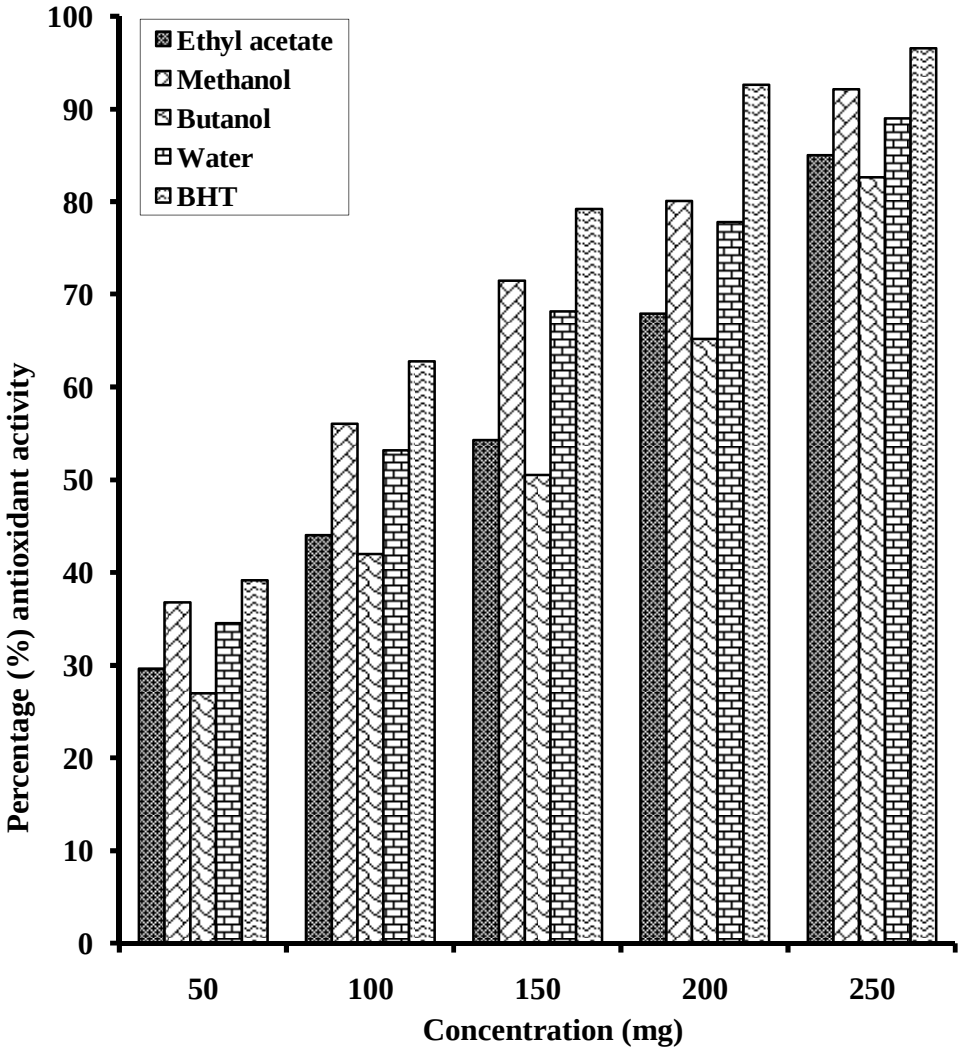

Figure 2. Antioxidant activity of celery extracts and BHT at different concentrations as assayed by β-carotene-linoleate model system. Each sample was assayed in triplicate for each concentration. Experimental results are means ± SD of three parallel measurements.

complex between high molecular weight phenolics and compounds). Those kinds of phenolic compounds show antioxidant activity due to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triple oxygen or decomposing peroxide. BHT showed higher degree of free radical scavenging activity than that of the extracts at low concentration points. The DPPH activity of BHT exhibited 92.04% at 50 µg/ml concentration.

Antioxidant activity using β- carotene-linoleate model system

The antioxidant activity through β-carotene-linoleate model system of extracts at 50 to 250 µg/ml concentrations was assayed and compared with BHT (Figure 2). The antioxidant activity of the extracts was found to increase with the increase in its concentration. For BHT, the increase of antioxidant activity was significant between 50 and 250 µg/ml and the same concentration was also significant with the extracts too. BHT was found to possess slightly higher degree of antioxidant activity than the extracts at each concentration points. At 250 µg/ml concentration, all the extracts and the BHT showed highest antioxidant activity (Figure 2), methanol extract (92.09%) followed by water (88.95%), ethyl acetate (84.98%) and butanol (82.59%), while the antioxidant activity of BHT was 96.51%, respectively. The addition of the extracts and BHT

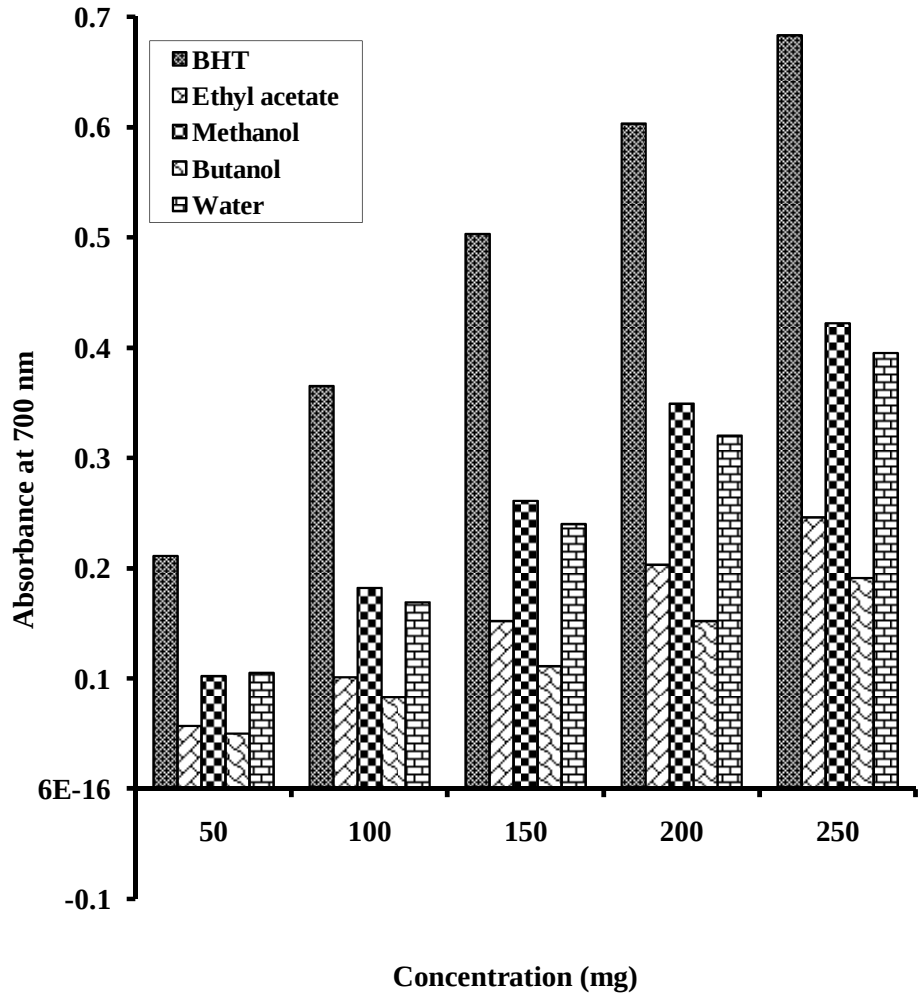

Figure 3. Reducing power of celery extracts and BHT at different concentration levels. Each sample was assayed in triplicate for each concentration. Experimental results are means ± SD of three parallel measurements.

between 50 and 250 µg/ml concentrations prevented the bleaching of β-carotene to different degrees.

These results may suggest that the extracts may be an indication of potential antioxidant. In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As a result, β-carotene gets oxidized and breaks down in part, subsequently the system loses its chromophore and characteristic orange color, which is monitored spectrophotometrically (Shon et al., 2003). The antioxidant activities of the extracts can be attributed to the mechanism: polyphenolic compounds associated in neutralizing the linoleate free radical and other free radicals formed in this model system, which oxidize unsaturated β-carotene molecules.

Assay of reductive potential

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities (Re et al., 1999). In the present study, the extracts and BHT exhibited effective reducing capacity at all concentration points. The reducing capacity of the extracts and BHT increased with increase in the concentration (Figure 3).

The reducing power of the extracts followed the

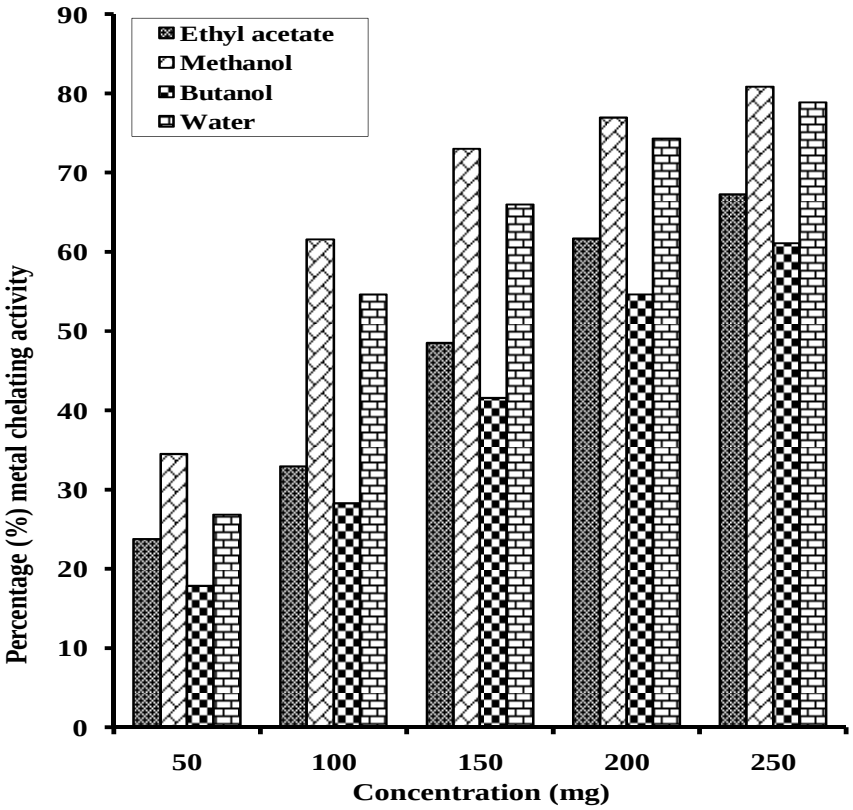

Figure 4. Metal ion chelating effect of celery extracts and BHT at different concentration levels. Each sample was assayed in triplicate for each concentration. Experimental results are means ± SD of three parallel measurements.

order –methanol < water < ethyl acetate < butanol. The reducing capacity of BHT was found to be higher than the extracts at each concentration points. Earlier authors (Gao et al., 2000; Amarowicza et al., 2004) have observed a direct correlation between antioxidant activities and reducing capacity of certain plant extracts. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing capacity of extracts suggested that reductone-associated and hydroxide groups of compounds can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions.

Chelating effects on ferrous ions

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red color of the complex. Measurement of color reduction therefore allows estimating the metal chelating activity of the coexisting chelator (Yamaguchi et al., 2000).

Metal chelating activity was claimed as one of the antioxidant activity mechanism, since it reduced the concentration of the catalyzing transition metal in lipid peroxidation (Senevirathne et al., 2006). It was reported that chelating agents, which form s-bonds with metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Keowmaneechai and McClements, 2006).
In this assay, both the extracts and standard compounds interfered with the formation of ferrous complex with the reagent ferrozine, suggesting that it has chelating activity and captures the ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex is linearly decreased with the dose taken (50 to 250 µg/ml). The percentages of metal chelating capacity at 250 µg/ml doses of the extracts were found to be methanol (80.85%), water (78.89%), ethyl acetate (67.25%) and butanol (61.07%), respectively (Figure 4). The standard EDTA exhibited 98.59% activity at 100 µg/ml

Table 2. Antioxidant capacity of *Apium graveolens* leaves extract by phosphomolybdenum method.

Extract	Antioxidant capacity (%) as equivalent to α-tocopherol (mg/g)
Ethyl acetate	47.50 ± 2.33
Methanol	75.25 ± 4.73
Butanol	73.01 ± 3.44
Water	74.29 ± 2.70

Values are means of triplicates ± SD.

concentration. Metal chelating activity was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh et al., 1999). The data obtained from Figure 4 reveals that the extracts demonstrate an effective capacity for iron binding, suggesting that its action as antioxidant may be related to its iron-binding capacity.

Evaluation of antioxidant capacity by phosphomolybdenum method

The antioxidant capacity of the extracts was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the extracts was found to decrease in the order, methanol > water > ethyl acetate > butanol extract (Table 2).

Conclusion

In our present study, the decreasing order of antioxidant activity among the *A. graveolens* leaves extracts assayed through all the methods was found to be methanol > water extract > ethyl acetate extract > butanol extract. This order is similar to the phenolic contents of the extracts that showed the extent of antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract.

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