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Antioxidant properties of methanolic extract from *Inula graveolens* L.

Adnan J. M. AL-FARTOSY

Department of Chemistry, College of Science, University of Basrah - IRAQ

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Abstract: The aim of this study was to assess the possible antioxidant activities of 80% methanolic extract from the *Inula graveolens* L. plant. It was found that the methanolic extract has the highest content of phenolic (1.63%, calculated as gallic acid) and flavonoid (0.52%, calculated as quercetin) equivalents per 100 g of dry mass. The methanolic extract possesses strong antioxidant activity (64.28%) as well as strong reducing power (increasing as the extract concentration increases) and ferrous ion chelating (96%) abilities. Moreover, the methanolic extract shows the highest free radical-scavenging activities for superoxide anion and hydroxyl radical, reaching 93.43% and 91.38%, respectively.

Key words: Antioxidant properties, ferrous ion chelating effect, hydroxyl radical scavenging activity, *Inula graveolens* L., reducing power, superoxide radical scavenging activity

Introduction

Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl, and nitric oxide radicals play an important role in oxidative stress related to the pathogenesis of various important diseases (Finkel and Holbrook 2000). Antioxidants are vital substances that possess the ability to protect the body from damage caused by free radical-induced oxidative stress. A variety of free radical scavenging antioxidants exist within the body, many of which are derived from dietary sources like fruits, vegetables, and teas (Souri et al. 2004). Medicinal plants, as sources of remedies, are widely used as alternative therapeutic tools for the prevention or treatment of many diseases (Harnafi and Amrani 2008). Flavonoids and phenolic compounds are widely distributed in plants that have been reported to exert multiple biological effects including antioxidant, free radical scavenging, anti-inflammatory, and anticarcinogenic (Miller 1996). There is limited information published on the biological activities of the *Inula graveolens* L. plant. Hence, in the present study, an attempt was made to investigate the possible antioxidant activities of the methanolic extract of *Inula graveolens* (MEIG) through various in vitro models.

Materials and methods

Plant material

The *Inula graveolens* L. plant used in this study, was collected in October 2009 from the Abu-Al-Khaseeb region (southern Basrah), Iraq. The plant was botanically authenticated and 3897 voucher specimens were deposited in the Herbarium of Basrah (Iraq, Basrah, College of Science, University of Basrah).

* E-mail: alfartosy_abuhaneen@yahoo.com

Chemicals

All of the chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and the solvents were from E. Merck (Darmstadt, Germany). All of the reagents were prepared in deionized distilled water to eliminate the contamination of metal ions.

Extraction

A published method by Elmastas et al. (2006) was adopted. A quantity (100 g) of powdered plant was extracted in a Soxhlet apparatus with 80% methanol, for 24 h. The methanolic extract was filtered and evaporated to dryness under reduced pressure in a rotary evaporator to afford 9.47 g of dry extract.

Total phenolic content

The total phenolic content of the *Inula graveolens* L. extract was determined according to Hagerman et al. (2000) with a slight modification (Gülçin et al. 2007). To 0.5 mL of sample (0.25 mg mL^{-1}), 7 mL of ddH_2O, 0.25 mL of folin-Ciocalten reagent, and 1.25 mL of 20% aqueous sodium carbonate solution were added and mixed well. After incubation for 45 min in the dark, the absorbance was spectrophotometrically recorded at 725 nm versus the blank (0.5 mL of sample was replaced by 0.5 mL of 80% methanol solvent). The result was expressed as percentage of gallic acid equivalents (GAE) per 100 g dry mass using a standard calibration curve of gallic acid.

Total flavonoid assay

The total flavonoid concentration was measured by the aluminum chloride colorimetric assay (Marinova et al. 2005). The methanolic extract (0.5 mg mL^{-1}) was added to a 10 mL volumetric flask containing 4 mL of ddH_2O. To the above mixture, 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After 6 min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with ddH_2O. The solution was mixed well and the absorbance was measured at 510 nm against a blank (1 mL of sample was replaced by 1 mL of 80% methanol solvent). The flavonoid content was expressed as percentage of quercetin equivalent per 100 g dry mass.

Determination of antioxidant activity

The antioxidant activity of the methanolic extract was determined according to the β-carotene bleaching method developed by Karadeniz et al. (2005). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform; 1 mL of this solution was then pipetted into a round-bottom rotary flask containing 20 mg of linoleic acid and 0.2 g of Tween 20. After removing the chloroform by vacuum evaporation using a rotary evaporator at 30 °C, 50 mL of aerated distilled water were added to the flask with manual shaking. Aliquots (5 mL) of this prepared emulsion were transferred into tubes containing 0.2 mL of extract (50 mg L^{-1}) or butylated hydroxytoluene (BHT, 50 mg L^{-1}), which was used for the purpose of comparison. The control consisted of 0.2 mL of 80% methanol instead of the extract. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. The samples were then subjected to thermal autoxidation at 50 °C in a water bath. Subsequent absorbance readings were recorded at 15 min intervals until the color of the β-carotene in the control sample disappeared (105 min). The extent of inhibition of the absorbance is related to the concentration of antioxidant compounds. The sample was assayed in triplicate. The degradation rate of extracts was calculated according to zero order reaction kinetics. Antioxidant activity (AA) was calculated as percent of inhibition relative to the control using the following equation:

$$AA = [1 - (Ai - At)/(Ái - Át)] \times 100$$

where

Ai = Measured absorbance value of sample at zero time.

At = Measured absorbance value of sample after incubation (105 min) at 50 °C.

Ái = Measured absorbance value of control at zero time.

Át = Measured absorbance value of control after incubation (105 min) at 50 °C.

Measurement of reducing power

The reductive capability of the extract was quantified by the method of Gülçin (2009). The methanolic extract (2, 4, 8, 12, 16, 20 mg mL^{-1}) or BHT was mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, 1% potassium ferricyanide, and then incubated at 50 °C for 20 min. An equal volume of 1% trichloroacetic acid was added to the

mixture and centrifuged at 3000 × *g* for 10 min at room temperature. The upper layer of the solution was mixed with ddH_2O and 0.1% $FeCl_3$ with a ratio of 1:1:2, and the absorbance at 700 nm was measured. This assay was done in triplicate. Increased absorbance indicated increased reducing power.

Ferrous ion chelating effect

A published method by Gülçin (2010) was adopted. Reaction mixtures containing 0.1 mL of the methanolic extract at different concentrations (2, 4, 8, 12, 16, 20 mg mL^{-1}), 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine were incubated at 37 °C for 10 min. After adding 1.5 mL of ddH_2O to the mixture, the absorbance at 562 nm was measured. This assay was done in triplicate. The lower absorbance at 562 nm indicated a stronger chelating effect. The percentage of ferrous ion chelating ability is expressed by [1 – (test sample absorbance/blank sample absorbance)] × 100.

Superoxide radical scavenging activity

The effect of the methanolic extract on superoxide anion radicals was estimated according to the method described by Talaz et al. (2009). The reaction mixture containing 1 mL of riboflavin (3.3 × 10 mol L^{-1}), methionine (0.01 mol L^{-1}), 1 mL of nitro blue tetrazolium chloride (NBT, 4.6 × 10 mol L^{-1}), and the solvent of the above solutions was phosphate buffer solution (0.05 mol L^{-1}, pH 7.8). After adding 1 mL of sample of different concentrations (2, 4, 8, 12, 16, 20 mg mL^{-1}), the reaction mixture was illuminated at 4000 lx and 25 °C for 30 min. Then the absorbance of the reaction mixture was measured at 560 nm with a spectrophotometer and the scavenging percentage was calculated according to the following formula:

Scavenging percentage = $(A_0 - A) \times 100/A_0$

where A_0 is the absorbance of the control without a sample. A is the absorbance with a sample.

Hydroxyl radical scavenging activity

Hydroxyl radicals were generated by a Fenton reaction system and the scavenging capacity towards the hydroxyl radicals was measured by using a deoxyribose method (Halliwell et al. 1987). The reaction mixture contained 0.8 mL of phosphate buffer solution (50 mmol L^{-1}, pH 7.4), 0.2 mL of a sample of different concentrations (2, 4, 8, 12, 16, 20 mg mL^{-1}), 0.2 mL of EDTA (1.04 mmol L^{-1}), 0.2 mL of $FeCl_3$ (1 mmol L^{-1}), and 0.2 mL of 2-deoxyribose (60 mmol L^{-1}). The mixtures were kept in a water bath at 37 °C and the reaction was started by adding 0.2 mL of ascorbic acid (2 mmol L^{-1}) and 0.2 mL of H_2O_2 (10 mmol L^{-1}). After incubation at 37 °C for 1 h, 2 mL of cold thiobarbituric acid (10 g L^{-1}) was added to the reaction mixture followed by 2 mL of HCl (25%). The mixture was heated at 100 °C for 15 min and then cooled down with water. The absorbance of solution was measured at 532 nm with a spectrophotometer. The hydroxyl radical scavenging capacity was evaluated with the inhibition percentage of 2-deoxyribose oxidation on hydroxyl radicals. The scavenging percentage was calculated according to the following formula:

Scavenging percentage = $[A_0 - (A_1 - A_2)] \times 100/A_0$

where A_0 is the absorbance of the control without a sample.

A_1 is the absorbance after adding the sample and deoxyribose.

A_2 is the absorbance of the sample without deoxyribose.

Dry matter determination

A published method by Chodak and Tarko (2007) was adopted. The sample was dried at 105 °C until the constant weight was obtained, cooled in a desiccator and weighed in an analytical scale. The dry matter was calculated by the following formula:

Dry matter = C – A/B – C

where A = The weight of empty weighing bottle, g.

B = The weight of weighing bottle with fresh sample, g.

C = The weight of weighing bottle with dried sample, g.

Results

Total phenolic and flavonoid contents

The total phenolic content was 1.63% of gallic acid equivalent, while the total flavonoid content was 0.52% of quercetin equivalent of dry mass of plant extract.

Antioxidant activity

Figure 1 shows the antioxidative activities of the methanolic extract compared to BHT. The extract of *Inula graveolens* L. showed the highest antioxidant activity (64.28%) at the concentration 50 mg L^{-1}. At the same time BHT at a concentration of 50 mg L^{-1} showed 88.57% inhibition.

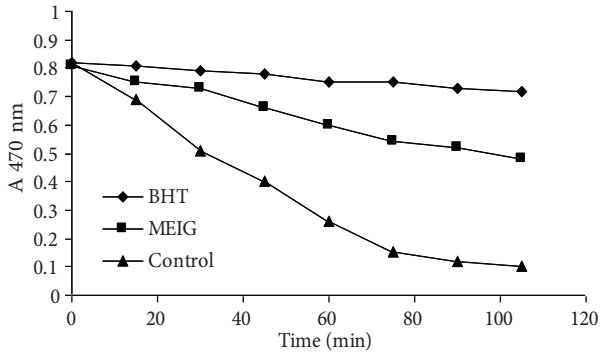

Figure 1. Antioxidant activity of the methanolic extract of *Inula graveolens* (MEIG) in comparison with BHT and control.

Reducing power

Figure 2 shows the reductive capabilities of the methanolic extract compared to BHT. The reducing power of extract of *Inula graveolens* L. was very potent and the reducing power of the extract was increased with quantity of sample. The plant extract could reduce the most Fe^{+3} ions, which had a lesser reductive activity than the standard of BHT.

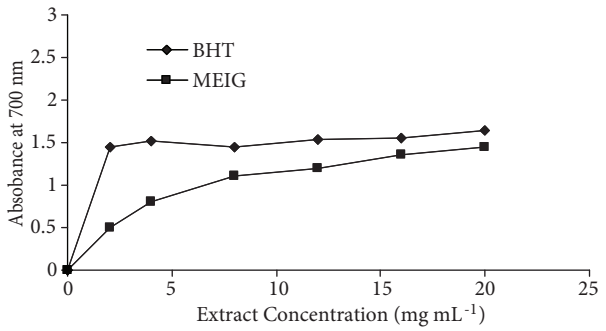

Figure 2. Reducing power of the methanolic extract of *Inula graveolens* (MEIG) in comparison with BHT.

Ferrous ion chelating ability

The ability of ferrous ion chelating by the methanolic extract was increased in a dose dependent manner, as illustrated in Figure 3. The extract of *Inula graveolens* L. at 2 mg mL^{-1} could reach more than (70%) ferrous ion chelating ability, while the best effect (up to 96%) was observed at a higher concentration (20 mg mL^{-1}). At the same time α-tocopherol hardly carried chelating ability.

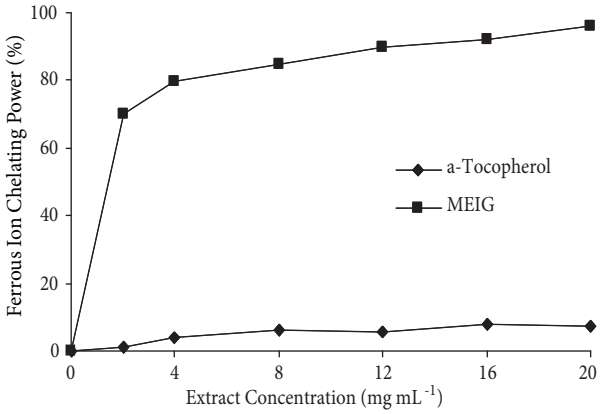

Figure 3. Ferrous ion chelating effect of the methanolic extract of *Inula graveolens* (MEIG) in comparison with α-tocopherol.

Superoxide radical scavenging activity

The scavenging of superoxide anion radicals was increased by raising the methanolic extract concentration of *Inula graveolens* L. from 4 to 12 mg mL^{-1}, as illustrated in Figure 4. The scavenging percentage was 82.51% at a concentration of 4 mg mL^{-1} and 93.43% at a concentration of 12 mg mL^{-1}. There was no significant effect on the scavenging capacity towards the superoxide anion radicals with a further increase in the concentration.

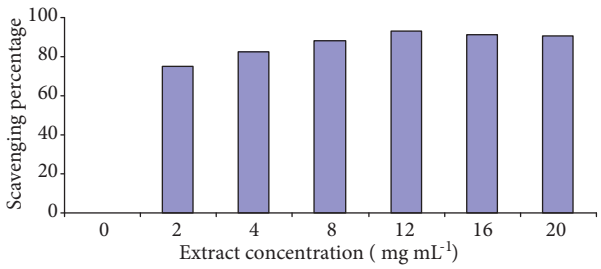

Figure 4. Scavenging effect of the methanolic extract of *Inula graveolens* (MEIG) on superoxide anion radicals.

Hydroxyl radical scavenging activity

The scavenging of hydroxyl radicals by the methanolic extract was increased in a dose dependent manner, as illustrated in Figure 5. The scavenging

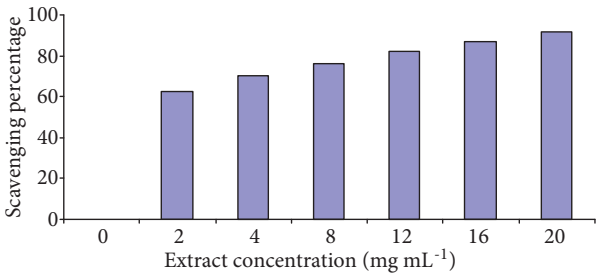

Figure 5. Scavenging effect of the methanolic extract of *Inula graveolens* (MEIG) on hydroxyl radicals.

percentage achieved 91.38% at a concentration of 20 mg mL^{-1}.

Discussion

The antioxidant activity of *Inula graveolens* L. methanolic extract could be attributed to its higher content of both phenolic and flavonoid compounds. Flavonoids act as scavengers of various oxidizing species, i.e. superoxide anion ($O_2^{\cdot -}$), hydroxyl radical, or peroxy radicals. They also act as quenchers of singlet oxygen (Das and Ratty 1986). Numerous plant constituents have been proven to show free radical scavenging or antioxidant activity (Aruoma and Cuppett 1997). Phenols are very important plant constituents. There is a highly positive relationship between the total phenols and antioxidant activity of many plant species, because of the scavenging ability of their hydroxyl groups (Vinson et al. 1998). It is also reported that phenolic compounds are effective hydrogen donors, making them very good antioxidants (Yen et al. 1993). However, the components responsible for the antioxidative activity of *Inula graveolens* L. are currently unclear. Therefore, further work must be carried out to isolate and identify these components.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al. 1995). The antioxidant activities of putative antioxidants have been attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued proton abstraction, and radical scavenging (Diplock 1997). The reducing power of the methanolic extract increased with increasing concentration of the extract, indicating that some compounds in the methanolic extract were electron donors and could also react with free radicals to convert them into more stable products and to terminate radical chain reactions.

A-tocopherol barely exhibited the ability of chelating ferrous ions due to its chemical structure properties. In the current study, the methanolic extract at 2 mg mL^{-1} could reach more than (70%) ferrous ion chelating ability, while the best effect (up to 96%) was observed at a higher concentration (20 mg mL^{-1}). In comparison with the test results reported by Wong and Yen (1997), the methanolic extracts of mungbean sprouts and radish sprouts only exhibited a chelating ability of 60% and 40%, respectively, at a concentration of 3 mg mL^{-1}. Therefore, domestic *Inula graveolens* L. is estimated to possess a higher ferrous ion chelating ability than mungbean and radish sprouts.

Superoxide anion is an initial free radical and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, or singlet oxygen in living systems (Stief 2003). It can also react with nitric oxide and from peroxynitrite, which can generate toxic compounds such as hydroxyl radicals and nitric dioxide (Halliwell 1997). The superoxide anion radical scavenging activity of the methanolic extract was assayed using an illuminating riboflavin system. Superoxide anion radicals reduce NBT to a blue colored formazan that is measured at 560 nm. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anions in the reaction mixture. The methanolic extract of *Inula graveolens* L. had strong superoxide radical scavenging activity. Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and immense biological damage (Kumar et al. 2008). It can react with lipids, polypeptides, saccharides, nucleotides, and organic acids, especially thiamine and guanosine, thereby causing cell damage (Jiao et al. 2005). The deoxyribose method is a simple assay to determine the rate constants for reactions of hydroxyl radicals (Halliwell et al. 1987). When the mixture of $FeCl_3$-EDTA, H_2O_2, and ascorbate were incubated with deoxyribose in a phosphate buffer (pH 7.4), the hydroxyl radicals generated an attack on the deoxyribose and result

in a series of reactions that cause the formation of MDA. Any hydroxyl radical scavenger added to the reaction would compete with the deoxyribose for the availability of hydroxyl radicals, thus reducing the amount of MDA formation. In the present study, the methanolic extract exhibited a strong scavenging capacity towards the hydroxyl radicals generated by the Fenton reaction in the test concentration range and the scavenging effects were increased with increasing concentration of the extract.

Conclusion

This study suggested that the methanolic extract of *Inula graveolens* L. possesses antioxidant activity that might be helpful in preventing or slowing the progress of various oxidative stress related diseases. Further investigation on the isolation and identification of antioxidant components in the plant may lead to chemical entities with the potential for clinical use.

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