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Antioxidant Activity of Phenolic Rich Fraction Obtained from *Convolvulus arvensis* L. Leaves Grown in Egypt

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ABSTRACT

Acidic ethyl acetate fraction was prepared from the leaves of *Convolvulus arvensis* L. contents of total phenolics and total flavonoids, antioxidant activity and reducing power were evaluated in this fraction. Total phenolics and total flavonoids were measured as 244.6±2.9 and 174.4±0.4 mg gallic acid and rutin equivalents per gram extract, respectively. The fraction exhibited strong antioxidant activity measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and its IC₅₀ value was 66.9±0.1 µg mL⁻¹. Further it possessed strong reducing power and inhibited the oxidation of β-carotene. GC-MS and HPLC analyses indicated that, this fraction contained a variety of phenolic compounds including p-hydroxybenzoic acid, syringic acid, vanillin, benzoic acid and ferulic acid. This study revealed that acidic ethyl acetate fraction of *C. arvensis* L. leaves has strong antioxidant activity which is correlated with its high content of phenolic compounds and it may serve as a phenolic rich fraction in food industry.

Key words: *Convolvulus arvensis* L., antioxidant activity, total phenolics, total flavonoids

INTRODUCTION

Reactive Oxygen Species (ROS) have been considered to cause oxidative injury to living organisms and thus play an important role in many human diseases such as arthritis, atherosclerosis, emphysema, aging process, diabetes mellitus and cancer (Elzaawely *et al.*, 2005; Gupta *et al.*, 2007; Juan and Chou, 2010). Synthetic antioxidants such as Tert-butyl Hydroxyanisole (BHA) and Tert-butyl Hydroxytoluene (BHT) are used to protect foods against oxidative damage caused by ROS (Liu *et al.*, 2011). However, these synthetic antioxidants have been proved to have undesirable side effects; therefore, there has been an increasing interest in the substitution of synthetic antioxidants by natural ones (Al-Soqeer, 2011). Phenolic compounds are natural plant-derived substances that have positive antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory and antimicrobial activities (Su *et al.*, 2009; Caliskan and Polat, 2011; Anago *et al.*, 2011).

Convolvulus arvensis (Family: Convolvulaceae) is a wild plant native to Europe and Asia and widely distributed in Egypt. It is a climbing or creeping herbaceous perennial plant growing to 0.5-2 m high. It showed an allelopathic activity (Om *et al.*, 2002; Hegab and Ghareib, 2010), antibacterial activity (Sener *et al.*, 1998) and antioxidant activity (Thakral *et al.*, 2010). Previous

phytochemical studies revealed that *C. arvensis* contained tropane alkaloids (Molyneux *et al.*, 1993; Todd *et al.*, 1995) and phenolic compounds (Hegab and Ghareib, 2010).

This study was designed to prepare a phenolic rich fraction from the leaves of *C. arvensis* grown in Egypt and to investigate its antioxidant capacity as well as its contents of phenolic compounds.

MATERIALS AND METHODS

Chemicals: Standard phenolic compounds (p-hydroxybenzoic acid, syringic acid, vanillin, benzoic acid and ferulic acid), Folin- Ciocalteu's reagent, aluminum chloride, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Tert-butyl Hydroxytoluene (BHT), β -carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), potassium ferricyanide, trichloroacetic acid, ferric chloride and all solvents used were of analytical grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Plant material: *C. arvensis* plants were collected from Tanta province, Egypt. The leaves were air dried for one week and the dried materials were powdered and kept in the refrigerator till use.

Preparation of the acidic fraction: Five grams dried powdered leaves of *C. arvensis* were extracted with aqueous ethanol 80% (3×200 mL) for 24 h at room temperature. The extracts were collected, filtered and the volume was reduced under vacuum at 40°C. After the pH of the solution adjusted to 11.0 by NaOH 4 N, it was extracted with chloroform (3×150 mL) to remove basic material that mainly contains alkaloids. The remaining aqueous solution was made acidic to pH 2.0 with HCl 6 N and then extracted with ethyl acetate (5×150 mL). The ethyl acetate fraction was collected, filtered and dried under vacuum at 40°C to yield 0.17 g of crude acidic fraction. The fraction was hydrolyzed with 50 mL NaOH 4 N at 50°C with stirring for 4 h to release phenolic acids. After the pH was adjusted to 2.0 by HCl 6 N, it was extracted with ethyl acetate (2×150 mL). The acidic ethyl acetate fraction was filtered and dried under vacuum to yield 0.13 g of phenolic-rich ethyl acetate fraction of *C. arvensis* leaves.

Determination of total phenolic content: The amount of total phenolics was determined according to the Folin-Ciocalteu procedure described by Kahkonen *et al.* (1999). Briefly, 1.0 mL Folin-Ciocalteu's reagent (50%) and 0.8 mL 7.5% (w/v) Na₂CO₃ were added to 0.2 mL (500 ppm) of methanolic solution of EtOAc fraction. After shaking, the mixture was incubated at room temperature for 30 min. Absorption was measured at 765 nm using a Shimadzu UV-160A spectrometer, Kyoto (Japan). Total phenolic content was expressed as Gallic Acid Equivalents (GAE) in milligrams per gram extract.

Determination of total flavonoids: The amount of total flavonoids was determined according to the method described by Djeridane *et al.* (2006). Briefly, 1.0 mL of methanolic solution of EtOAc fraction (1000 ppm) was mixed with 1 mL aluminum chloride (2% in methanol). After shaking, the mixture was incubated at room temperature for 15 min and then the absorption was measured at 430 nm using a Shimadzu UV-160A spectrometer, Kyoto (Japan). Total flavonoids content was expressed as Rutin Equivalents (RE) in milligrams per gram extract.

Antioxidant activity by DPPH method: The radical scavenging activity was evaluated as described previously by Abe *et al.* (1998). Two millileter of the methanol solution of the sample (25, 50 and 100 ppm) were mixed with 1 mL of 0.5 mM DPPH methanol solution and 2 mL of

0.1 M sodium acetate buffer (pH 5.5). After shaking, the mixture was incubated at room temperature in the dark for 30 min and then the absorbance was measured at 517 nm using a Shimadzu UV-160A spectrometer, Kyoto (Japan). BHT (10 ppm) was used as positive reference while methanol was used as negative one. The IC_{50} value was determined as the concentration required to give 50% DPPH radical scavenging activity.

Antioxidant activity by β -carotene bleaching method: Antioxidant activity was evaluated according to the β -carotene bleaching method as reported by Siddhuraju and Becker (2003). β -Carotene (2.0 mg) was dissolved in 10 mL chloroform. One milliliter of the chloroform solution was mixed with 20 μ L linoleic acid and 200 mg Tween-40. The chloroform was evaporated under vacuum at 45°C, then 50 mL oxygenated water was added and the mixture was vigorously shaken. The emulsion obtained was freshly prepared before each experiment. An aliquot (250 μ L) of the β -carotene-linoleic acid emulsion was distributed in each of the 96-wells of the microtitre plates. Methanolic solutions (30 μ L) of the sample and BHT at 1000 ppm were added. An equal amount of methanol was used for control. The microtitre plates were incubated at 50°C and the absorbance was measured using a model MTP-32 microplate reader (Corona Electric, Ibaraki, Japan) at 492 nm. Readings of all samples were performed immediately at zero time and every 15 min up to 180 min.

Reducing power: Reducing power was determined as described previously by Yildirim *et al.* (2003). One milliliter of the ethyl acetate fraction of *C. arvensis* leaves and BHT at different concentrations in methanol was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (10 g L^{-1}), then the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 mL trichloroacetic acid (100 g L^{-1}) was added to the mixture which was subsequently centrifuged at 4000 rpm for 10 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (1 g L^{-1}) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

GC-MS analysis: A 1 μ L aliquot of the acetone solution of the ethyl acetate fraction was injected into the GC-MS (QP-2010, Shimadzu Co., Kyoto, Japan). The DB-5MS column was 30 m in length, 0.25 mm id and 0.25 μ m in thickness (Agilent Technologies, J and W Scientific Products, Folsom, CA, USA). The carrier gas was helium. The GC oven temperature program was as follows: 50°C hold for 6 min, raised at 5°C min^{-1} to 280°C and hold for 5 min. The injector and detector temperatures were set at 250 and 280°C, respectively. The mass range was scanned from 20 to 900 amu. The control of the GC-MS system and the data peak processing were carried out by means of Shimadzu's GC-MS solution software, version 2.4.

Quantification by HPLC: Phenolic compounds were measured at 280 nm using a Shimadzu HPLC (SCL-10 A vp, Shimadzu Co., Kyoto, Japan) coupled with a UV-vis detector (SPD-20A, Shimadzu). Separations were achieved on a RP-18 ZORBAX ODS column (Agilent Technologies, USA) ($25 \times 0.46\text{ cm i.d.}$; 5 μ m particle size). The mobile phase was water with 1% acetic acid (v/v) (solvent A) and methanol:acetonitrile:acetic acid (75:24:1, v/v/v) (solvent B) at a flow rate of 0.8 mL min^{-1} . The gradient elution was performed as follows: 0-2 min, 5% B isocratic; 2-10 min, linear gradient 5-25% B; 10-20 min, linear gradient 25-40% B; 20-30 min, linear gradient 40-50% B; 30-40 min, linear gradient 50-100% B; 40-45 min, 100% B isocratic and 45-55 min, linear

gradient 100-5% B. A 5 μ L Methanolic solution of the ethyl acetate fraction at 10,000 ppm was used and the identification of the compounds was carried out by comparing their retention times to those of standards. The quantification of each compound was determined based on peak area measurements which were reported to calibration curves of the corresponding standards.

Statistical analysis: Data were analyzed using SAS version 6.12 using ANOVA with the Least Significant Difference (LSD) at the 0.05 probability level.

RESULTS AND DISCUSSION

Total phenolic and total flavonoids: Contents of total phenolic compounds were determined using Folin-ciocalteau method and total flavonoids determined using the aluminum chloride colorimetric assay of the acidic ethyl acetate fraction prepared from *C. arvensis* leaves are shown in Fig. 1. Results show that, this fraction contained high amounts of total phenolics and total flavonoids and they were measured as 244.6 ± 2.9 and 174.4 ± 0.4 mg gallic acid and rutin equivalents per g extract, respectively. It has been recorded that phenolic compounds including flavonoids are associated with strong antioxidant activity (Liu *et al.*, 2011) and they possess healthy benefits (Shih and Diagle, 2003).

Antioxidant activity: The DPPH radical scavenging activity of acidic ethyl acetate fraction of *C. arvensis* leaves and BHT as positive control are presented in Fig. 2. The IC_{50} defined as the concentration of the sample needed to scavenge 50% of DPPH present in the test solution. Lower

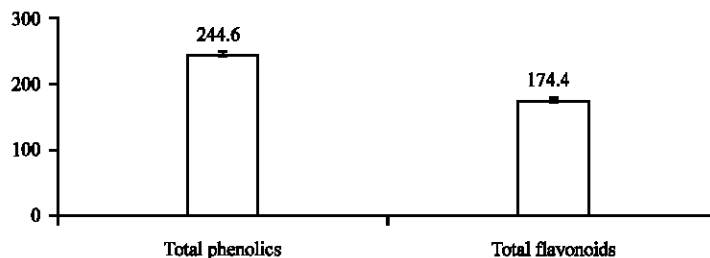


Fig. 1: Total phenolics (mg GAE g^{-1} extract) and total flavonoids (mg RE g^{-1} extract) in acidic ethyl acetate fraction of *C. arvensis* leaves

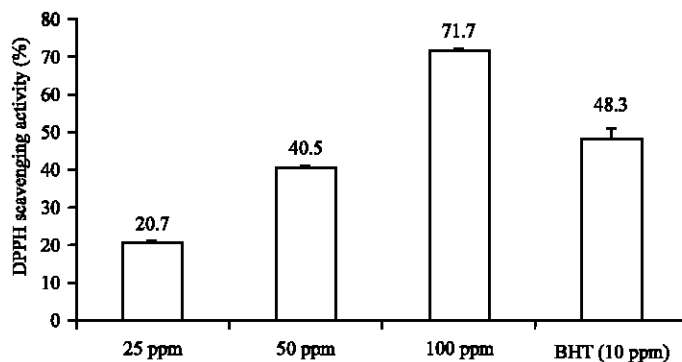


Fig. 2: DPPH radical scavenging activity of acidic ethyl acetate fraction of *C. arvensis* leaves and BHT

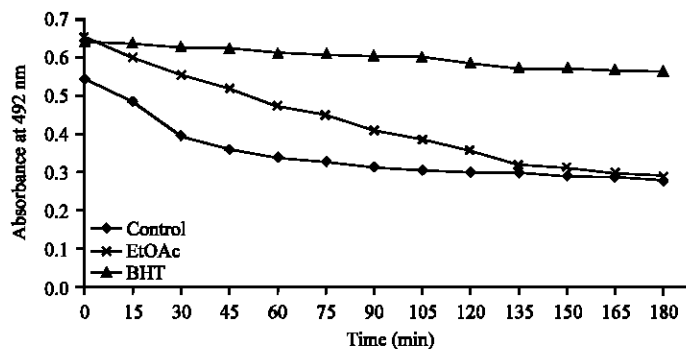


Fig. 3: Antioxidant activity of acidic ethyl acetate fraction of *C. arvensis* leaves and BHT measured by β -carotene bleaching method

IC₅₀ value reflects higher DPPH radical scavenging activity. DPPH is a stable radical showing a maximum absorbance at 515 nm. It can be reduced by an antioxidant molecule to uncolored solution (Erkan *et al.*, 2008). It is a convenient, accurate and easy method and therefore, it is widely used to measure the antioxidant activity of plant extracts (Blois, 1958; Ali *et al.*, 2011). Under the assay conditions employed here, the acidic ethyl acetate fraction of *C. arvensis* leaves showed strong DPPH activity with the IC₅₀ value of 66.9±0.1 $\mu\text{g mL}^{-1}$; while the IC₅₀ of BHT as standard was 12.2 $\mu\text{g mL}^{-1}$. This high activity may be due to the high amounts of the corresponding phenolic compounds found in this fraction. Positive correlation has been demonstrated between antioxidant activity and phenolic content of plant extracts (Su *et al.*, 2008; Biglari *et al.*, 2008; Socha *et al.*, 2009; Juan and Chou, 2010).

Effect of the acidic ethyl acetate fraction of *C. arvensis* leaves as well as BHT on oxidation of β -carotene/linoleic acid is shown in Fig. 3. 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 β -carotene molecules lose their double bonds by oxidation, the compound loses its orange color. The presence of extract/fraction that has antioxidant activity can hinder the extent of β -carotene-bleaching by neutralizing the free radicals formed in the system and thus, the degradation rate of β -carotene depends on the antioxidant activity of the extract/fraction (Jayaprakasha *et al.*, 2001). In present study, the acidic ethyl acetate fraction of *C. arvensis* leaves reduced the oxidation of β -carotene to some extent and its effect was less than that of BHT.

Reducing power is considered to be a strong indicator of the antioxidant activity and was determined using a modified iron (III) to iron (II) reduction assay. The presence of the reductants (antioxidants) in the solution can reduce the Fe³⁺/ferricyanide complex to the ferrous form by donating an electron (Ali *et al.*, 2011; Liu *et al.*, 2011). The reducing power of the acidic ethyl acetate fraction of *C. arvensis* leaves as well as standard BHT is presented in Fig. 4. The reducing power of both samples increased with its concentrations. The ethyl acetate fraction had high ability to reduce Fe³⁺ to Fe²⁺ and the effect was less than that of BHT. The reducing ability is generally associated with the presence of reductants which exert antioxidant action through breaking the free radical chain by donating a hydrogen atom or preventing peroxide formation (Kumaran and Karunakaran, 2006). The results presented in Fig. 4 indicated that the reducing activity of the ethyl acetate fraction attend to be due to the presence of phenolic compounds that may act as reductants by donating the electrons and reacting with free radicals to convert them to more stable products and terminate radical chain reaction (Loganayaki *et al.*, 2011).

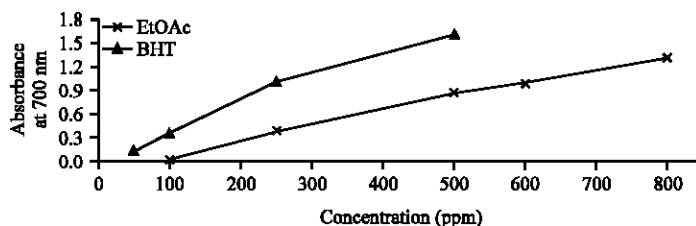


Fig. 4: Reducing powers of acidic ethyl acetate fraction of *C. arvensis* leaves and BHT

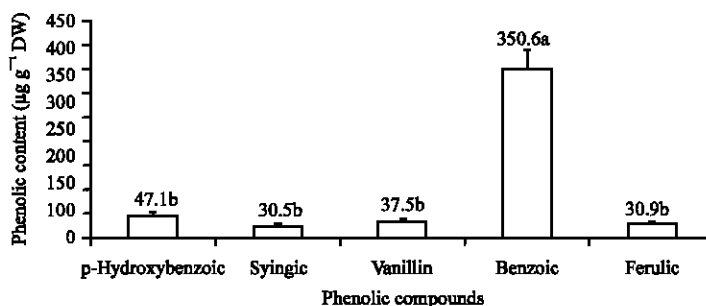


Fig. 5: Contents of phenolic compounds ($\mu\text{g g}^{-1}$ DW) in acidic ethyl acetate fraction of *C. arvensis* leaves LSD ($p < 0.05$) = 62.656. Values are means of 3 replications \pm SE. Means followed by different letters are significantly different at $p < 0.05$

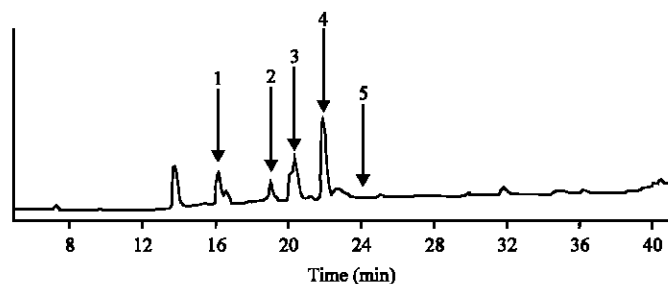


Fig. 6: HPLC chromatogram of phenolic compounds from acidic ethyl acetate fraction of *C. arvensis* leaves. 1: p-Hydroxybenzoic, 2: Syringic, 3: Vanillin, 4: Benzoic and 5: Ferulic

Identification and quantification of phenolic compounds: Since phenolic acids are often found in plants in bound complexes such as esters and glycosides (Germano *et al.*, 2006), it needs to be released from its bound form by using alkaline, acidic or enzymatic hydrolysis (Madhujith and Shahidi, 2009). In this study, alkaline hydrolysis with 4 M NaOH at 50°C was carried out to release conjugated phenolics and subsequently five phenolic compounds were detected in the acidic ethyl acetate fraction of *C. arvensis* by GC-MS and HPLC. These compounds are p-hydroxybenzoic acid, syringic acid, vanillin, benzoic acid and ferulic acid. Figure 5 displays quantitative results of identified phenolic compounds in the acidic ethyl acetate fraction of *C. arvensis* by HPLC and Fig. 6 shows the representative HPLC chromatogram of this fraction monitored at 280 nm. The quantification of the compounds using HPLC was carried out by comparing their retention times to those of standards. Furthermore, the identification of the compounds was confirmed using the GC-MS by comparing their retention times and spectral characteristics of their peaks with those of

standards. Statistically significant variation was found in the quantities of phenolic compounds in this fraction. Benzoic acid was the predominant phenolics ($350.6 \mu\text{g}^{-1}$ DW) while syringic acid was the lowest one ($30.5 \mu\text{g}^{-1}$ DW). Previous study reported that the methanol extract and its ethyl acetate fraction prepared from the aerial parts of *C. arvensis* grown in India showed strong antioxidant activity; however, its chemical constituents were not identified (Thakral *et al.*, 2010). It was previously reported that phenolic compounds have strong antioxidant activity (Elzaawely *et al.*, 2007; Zhang *et al.*, 2009; Mariod *et al.*, 2010) and therefore, the phenolic compounds in the phenolic rich fraction of *C. arvensis* may be responsible for its antioxidant activity.

CONCLUSIONS

This investigation shows that, the acidic ethyl acetate fraction prepared from *C. arvensis* leaves contains high amounts of total phenolics and total flavonoids and it exhibited strong reducing power and antioxidant activity measured by DPPH and β -carotene bleaching methods. GC-MS and HPLC revealed that this fraction contains five phenolic compounds viz., p-hydroxybenzoic acid, syringic acid, vanillin, benzoic acid and ferulic acid that are responsible for its strong antioxidant activity. Further studies in isolation of individual phenolic compounds particularly flavonoids in this fraction and its effect on antioxidant status in animal models are needed to evaluate their potential benefits.

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