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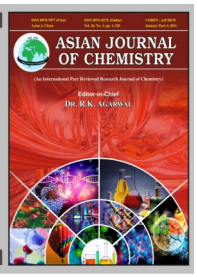

Investigation on the Antioxidant Activity of Roots and Stem of *Colchicum turcicum* L.

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Water and acetone extracts from *Colchicum turcicum* L. were investigated for their antioxidant and radical scavenging activities in four different assays, namely, total antioxidant activity, 1,1-dipenyl-2-picryl-hydrazyl (DPPH) free radical scavenging, hydrogen peroxide scavenging and ferrous chelating activities. Antioxidant activity, ferrous chelating and hydrogen peroxide scavenging activities of *C. turcicum* extracts showed dose dependence and increased with concentration of *C. turcicum* extract. At the same concentration (100 µg/mL), water extract of *C. turcicum* (WECT) and acetone extract of *C. turcicum* (AECT) showed 53 and 48 % inhibition of lipid peroxidation of linoleic acid emulsion, respectively. On the other hand, butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid indicated inhibitions of 77, 71 and 59 % on the peroxidation of linoleic acid emulsion, respectively, at 50 µg/mL. Also, the total phenolic content in both water extract of *C. turcicum* and acetone extract of *C. turcicum* were determined as catechol equivalents. The total phenolics content of water and acetone extracts were determined by the Folin-Ciocalteu procedure and 0.454 and 2.172 mg catechol equivalent of phenols was detected in 1 g water extract of *C. turcicum* and acetone extract of *C. turcicum*.

Keywords: *Colchicum turcicum* L., Antioxidant activity, Total phenol content, Total flavonoids, DPPH.

INTRODUCTION

Lipid peroxidation is one of the major reasons for deterioration of food products during processing and storage. Radicals are known to take part in lipid peroxidation and play an important role in the progression of a large number of pathological disturbances, such as atherosclerosis, brain dysfunction, cancer promotion, heart diseases, immune system decline and neurodegenerative diseases[1].

Antioxidants are the compounds that, when added to food products, act as radical scavengers, prevent the radical chain reactions of oxidation, delay or inhibit the oxidation process and increase shelf life by retarding the process of lipid peroxidation[2].

Consumers are becoming more conscious of the nutritional value and safety of their food and ingredients. The preference for natural foods and food ingredients that are believed to be safer, healthier and less to hazards is increasing compared to their synthetic counterparts[3]. Thus, the evaluation of antioxidative activity of naturally occurring substances has been focus of interest in recent years[4]. The use of plants, herbs as antioxidants in processed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants[5].

Colchicum turcicum L. belongs to the Colchicaceae. Members of the Colchicaceae are perennial herbs with a subterranean corm or rhizome and hypogynous flowers with six petals[6]. *Colchicum* plants which a genus containing around 160 species are native to Balkans, North Turkey, Central Europe and North Africa[7]. They are source of colchicinoid alkaloids which possess different and valuable therapeutic activities. Colchicine possesses antiinflammatory properties as it is the drug of choice in the treatment of gout[8], familial Mediterranean fever[9] and Behcet's disease[10]. In addition, clinical studies have proved colchicine to possess a potent antitumor activity. Due to the lack of tumor selectivity and high toxicity, colchicine use as an antineoplastic drug is limited[11]. *Colchicum turcicum* L. is used as analgesic, antiinflammation, cell division and cell proliferation inhibitor. Determination of colchicine has been reported in *Colchicum* species by many researchers[12]. *Colchicum speciosum* (Colchicaceae) is used for its cholinergic activity[13]. Less information is available about antioxidative activity of these plants.

The antioxidant activities of *C. turcicum* extracts were evaluated based on the ability of the extracts to inhibit lipid peroxidation in linoleic acid emulsion, to scavenge DPPH and H_2O_2, to bind to Fe(II) ions. The results were compared to those of gallic acid, catechol, ascorbic acid, butylated hydroxyanisole and butylated hydroxytoluene.

EXPERIMENTAL

Linoleic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, sodium nitrite, aluminum nitrate and 3-(2-pyridyl)-5,6-*bis*(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate, ferrous chloride, polyoxyethylenesorbitan monolaurate (Tween-20), trichloroacetic acid, ethanol and acetone were purchased from Merck. All other chemicals used were in analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant material and extraction procedures: The roots and stem of *C. turcicum* were collected from mountains at Trabzon city (Tonya-Trabzon, Turkey). Plant materials were washed with distilled water and dried at room temperature. For water extraction, 25 g sample was put into a fine powder in a mill and was mixed with 500 mL boiling water by magnetic stirrer for 15 min. Then, the extract was filtered over Whatman No. 1 paper and the filtrate was collected, then water was removed by a rotary evaporator (Buchi R-200, Switzerland) at 40 °C to obtain dry extract. For acetone extraction, 25 g sample was put into a fine powder in a mill and was mixed with 500 mL acetone. The residue was re-extracted until extraction solvents became colourless. The obtained extracts were filtered over Whatman No. 1 paper and the filtrate was collected, then solvent was removed by a rotary evaporator (Buchi R-200, Switzerland) at 40 °C to obtain dry extract. Both of the extracts were kept at -20 °C and were dissolved in water or solvent before use.

Determination of total phenolics: Total phenols were determined according to the method described by Slinkard and Singleton[14]. About, 1 mL of extract solution containing 1 mg exracts in a volumetric flask was diluted with 46 mL of distilled water. About 1 mL of Folin-Ciocalteau reagent was added and mixed thoroughly. After 3 min, 3 mL of 2 % sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance of the blue colour that developed was measured at 760 nm. The concentration of total phenols was expressed as mg/g of dry extract[15]. The concentration of total phenolic compounds in the extract was determined as mg of catechol equivalent using the standard curve of catechol.

Determination of total flavonoids: Total flavonoid content was determined by a colorimetric method described by Wang *et al.*[16] with minor modification. An aliquot of 10 mL of appropriate dilution of each extract was added to volumetric flask containing 1 mL of 5 % (w/v) sodium nitrite and placed for 6 min, followed by reaction with 1 mL of (10 %) (w/v) aluminum nitrate to form a flavonoid-aluminum complex. After 6 min, 10 mL of 4.3 % (w/v) NaOH was added and the total was made up to 25 mL with distilled water. After 15 min at room temperature, the final solution was mixed well again and the absorbance was measured against a blank at 510 nm with a UV-1601 UV/VIS Recording Spectrophotometer (Shimadzu UV-1601, Japan). The concentration of total flavonoids was expressed as mg/g of dry extract. The concentration of total flavonoid compounds in the extract was determined as mg of gallic acid equivalent using an equation obtained from the standard gallic acid graph.

DPPH radical scavenging assay: The DPPH radical scavenging activities of *C. turcicum* extracts were evaluated by the method of Blois[17] with minor modifications. About, 0.1 mM solution of DPPH in ethanol was prepared and 1 mL of this solution was added to 3 mL of *C. turcicum* extracts at different concentrations (50-250 µg/mL). The reaction mixture was incubated for 0.5 h at 25 °C in the dark. The control contained all reagents without the sample and was used as blank. The DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm using a spectrophotometer. The DPPH radical scavenging activity (%) of the sample was calculated as:

$$1-\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\times 100$$

Determination of antioxidant activity in linoleic acid system: The total antioxidant activity of *C. turcicum* extracts was determined according to the thiocyanate method[18]. For stock solutions, 10 mg of each *C. turcicum* extracts was dissolved in 10 mL water (or solvent). Then, the solution, which contains the same amount of *C. turcicum* extracts (50 and 100 µg/mL) or standard samples (50 µg/mL) in 2.5 mL of 0.04 M potassium phosphate buffer (pH = 7) was added to 2.5 mL of linoleic acid emulsion in 0.04 M potassium phosphate buffer (pH = 7). Fifty milliliters linoleic acid emulsion contained 175 µg Tween-20, 155 µL linoleic acid and 0.04 M potassium phosphate buffer (pH = 7). On the other hand, 5 mL control was composed of 2.5 mL linoleic acid emulsion and 2.5 mL of 0.04 M potassium phosphate buffer (pH = 7). The mixed solution (5 mL) was incubated at 37 °C in a glass flask. The peroxide level was determined by reading the absorbance at 500 nm. This step was repeated every 12 h until the control reached its maximum absorbance value. The percent inhibition of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = \left(\frac{A_0 - A_1}{A_0}\right)\times 100$$

where A_0 is the absorbance of the control; A_1 is the absorbance of the sample.

Hydrogen peroxide scavenging assay: The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al.*[19]. For this purpose, a solution of H_2O_2 (40 mM) was prepared in phosphate buffer (0.1 M, pH = 7.4) and 0.6 mL of this solution was added to extracts (10-100 µg/mL) in phosphate buffer. The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contained the sodium phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of extracts and standard compounds was calculated as:

$$H_2O_2 \text{ scavenging activity (\%)} = \left(\frac{A_0 - A_1}{A_0}\right)\times 100$$

where, A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

Ferrous ions (Fe^{2+}) chelating activity: The chelation of ferrous ions by *C. turcicum* extracts was estimated by the

method of Dastmalchi *et al.*[20] with minor modifications, wherein the Fe^{2+}-chelating activity of extracts (50-250 µg/mL) was monitored by the absorbance of the ferrous iron-ferrozine complex at 562 nm. Extracts in 0.4 mL were added to a solution of 2 mM $FeCl_2$ (0.2 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.4 mL) and total volume was adjusted to 4 mL of ethanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by using the formula given bellow:

$$\text{Ferrous ions chelating efffect}\,(\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

where, A_0 is the absorbance of the control, A_1 is the absorbance of the sample

RESULTS AND DISCUSSION

Extract yield (amount of total extractable compounds) and contents of total phenolics and flavonoids: Water and acetone extracts prepared from 25 g *C. turcicum* gave a yield of 37.85 and 19.44 mg extractable compounds (EC) per gram of dry weight, respectively (Table-1).

Phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activities[21]. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteau reagent[22]. However, it should be noted that some chemical group of amino acids, proteins, organic acids, sugar and aromatic amines could react with the reagent[23]. In this study, the roots and stem of *C. turcicum* were dried before extraction while ascorbic acid was lost during drying process and amino acids, proteins and sugars can be removed from the extraction solvents. Thus, interference from ascorbic acid or other compounds like amino acids, proteins and sugars should be very little. This study exhibited that the total phenolic content per gram of crude extract of acetone extract (2.172 mg CE/g) was found to be higher than that of the water (0.454 mg CE/g), using the standard curve of catechol ($R^2 = 0.9992$) (Table-1).

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties[24]. The content of total flavonoids expressed as gallic acid equivalents, varied from 13.1 to 83.9 mg as GAE/g extract ($R^2 = 0.9978$) (Table-1).

DPPH radical scavenging activity: The capacity of extracts to scavenge the lipid-soluble DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, which result in the bleaching of the purple colour exhibited by the stable DPPH radical, is monitored at an absorbance of 517 nm. Positive DPPH test suggests that the samples were free radical scavengers. The effect of *C. turcicum* extracts and standard on DPPH radical was compared and shown in Fig. 1. The scavenging effect of *C. turcicum* extracts and standards on DPPH radical was compared. On the DPPH radical, *C. turcicum* extracts had significant scavenging effects with increasing concentration in the range of 50-250 µg/mL when compared with that of standards, the scavenging effect of *C. turcicum* extracts was lower. Ascorbic acid, butylated hydroxyanisole and butylated hydroxytoluene were used as standards for radical scavengers. The scavenging activity of water extract, acetone extract, ascorbic acid, butylated hydroxyanisole and butylated hydroxytoluene on DPPH radicals were 19, 54, 98, 92 and 90 % at a concentration of 250 µg/mL, respectively. *C. turcicum* extracts showed lower scavenging activity than the standards. Scavenging activity of extracts and standards decreased in order of ascorbic acid > butylated hydroxyanisole > butylated hydroxytoluene > acetone extract > water extract.

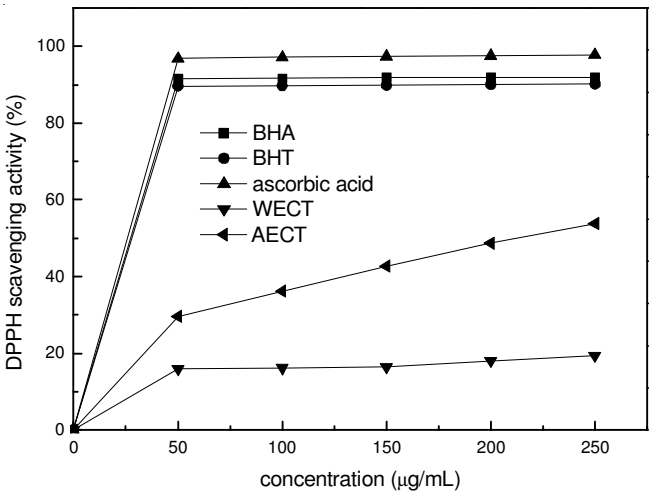

Fig. 1. DPPH free radical scavenging activity of different concentrations (50-250 µg/mL) of *C. turcicum* extracts and standard antioxidants: butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid

DPPH scavenging activity is best presented by EC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50 % of DPPH present in the test solution (Table-1). A higher DPPH radical scavenging activity was associated with a lower EC_{50} value. EC_{50} values for water extract, acetone extract, butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid on DPPH radical scavenging activity were found as 29.98, 45.74, 24.82, 24.70 and 25.54 µg/mL. *C. turcicum* extracts showed similar DPPH radical scavenging

TABLE-1
EXTRACTION YIELD, TOTAL PHENOLIC COMPOUNDS (TPC) (AS CATECHOL EQUIVALENTS), TOTAL FLAVONOIDS (TFC) (AS GALLIC ACID EQUIVALENTS AND ANTIOXIDANT EFFECT (EC_{50}) OF DPPH RADICALS AND FERROUS CHELATING POWER OF *C. turcicum* EXTRACTS

Extract	Extraction yield (%)	TPC (mg/g)	TFC (mg/g)	EC_{50} (µg/mL)	
				Scavenging ability on DPPH radicals	Chelating power
Water	37.85	0.454	13.1	29.98	116.74
Acetone	19.44	2.172	83.9	45.74	117.64

activities compared to the DPPH radical scavenging activity of the standards.

Total antioxidant activity determination: The ferric thiocyanate method measures the amount of peroxide, which is the primary product of oxidation produced during the initial stages of oxidation. *C. turcicum* extracts exhibited effective antioxidant activity in the linoleic acid emulsion system. At 100 µg/mL, the total antioxidant activity of water extract and acetone extract of *C. turcicum* was 53 % and 48 %, respectively (Fig. 2). This activity was lower than 50 µg/mL concentrations of ascorbic acid (59 %), butylated hydroxytoluene (71 %) and butylated hydroxyanisole (77 %). The peroxidation of linoleic acid emulsion without *C. turcicum* extracts or standard compounds was accompanied by a rapid increase of peroxides. Consequently, these results clearly indicate that *C. turcicum* extracts had effective and potent antioxidant activity in the ferric thiocyanate assays.

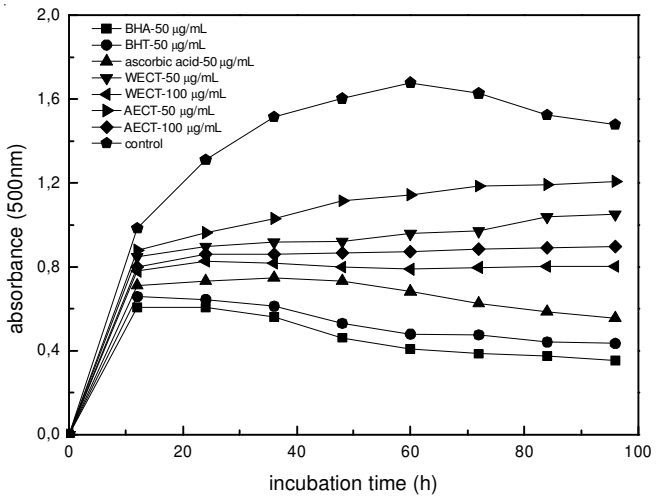

Fig. 2. Total antioxidant activities of different concentrations (50-100 µg/mL) of *C. turcicum* extracts and standard antioxidant compounds such as butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid at the concentration of 50 µg/mL

Hydrogen peroxide scavenging activity: Hydrogen peroxide can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. The ability of *C. turcicum* extracts to scavenge hydrogen peroxide is shown in Fig. 3 and compared with that of butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid as reference compounds. Hydrogen peroxide scavenging activity of water extract and acetone extract of *C. turcicum* at 100 µg/mL was found to be 29 and 27 %, respectively. On the other hand, butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid exhibited 31, 30 and 22 % hydrogen peroxide scavenging activity, respectively, at the same concentration. These results show that *C. turcicum* extracts have an effective hydrogen peroxide scavenging activity. At the above concentration, the hydrogen peroxide scavenging effect of extracts and three standard compounds decreased in order of butylated hydroxyanisole ≈ butylated hydroxytoluene ≈ water extract > aseton extract > ascorbic acid.

Ferrous chelation (II) activity: By forming a stable iron (II) chelate, an extract with high chelating power reduces the

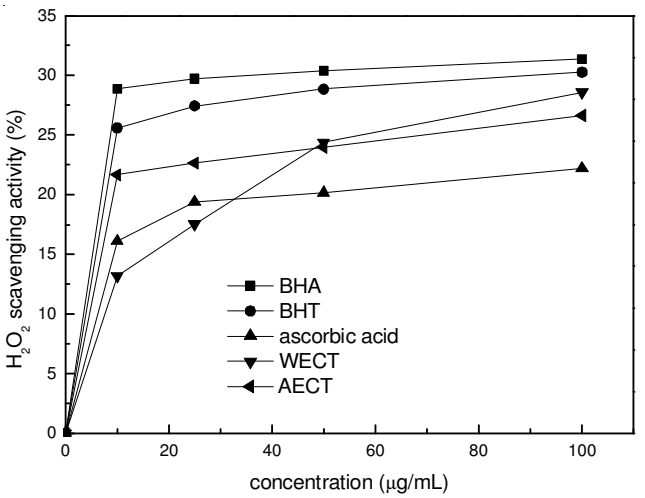

Fig. 3. H_2O_2 scavenging activity of different concentrations (10-100 µg/mL) of *C. turcicum* extracts and standard antioxidants: butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid

free ferrous ion concentration and thus decreases the extent of the Fenton reaction which is implicated in many diseases[25]. All the extracts demonstrated an ability to chelate ferrous (II) ions in a dose-dependent manner. As can be seen in Fig. 4, the ferrous ion chelating effect of *C. turcicum* extracts was compared to that of butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid. Water extract and acetone extract of *C. turcicum* exhibited 24 and 12.1 % chelation of ferrous ions at 250 µg/mL, respectively. On the other hand, the ferrous ion chelating capacities of the same concentration of butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid were found to be 32.4, 37.7 and 32.4 %, respectively.

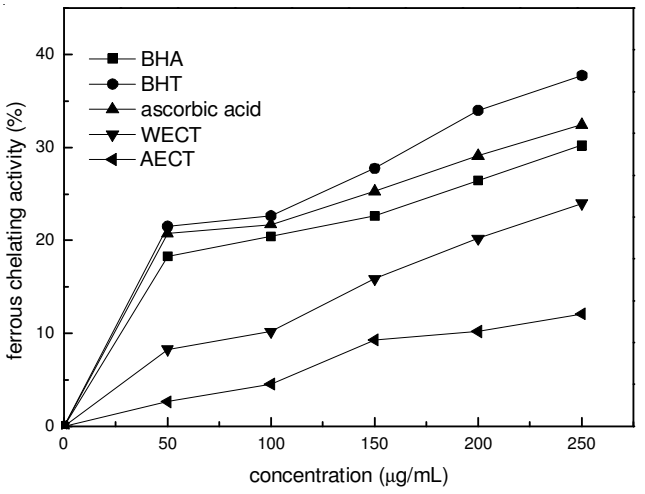

Fig. 4. Ferrous chelating activity of different concentrations (50-250 µg/mL) of *C. turcicum* extracts and standard antioxidants: butylated hydroxyanisole, butylated hydroxytoluene and ascorbic acid

From the estimated EC_{50} values, defined as the concentration of extract required to chelate 50 % of the available ferrous (II), it can be seen that the most effective ferrous (II) chelating was ascorbic acid (38.84 µg/mL), followed by butylated hydroxyanisole (42.98 µg/mL) and butylated hydroxytoluene (44.38 µg/mL), in decreasing order. These were significantly different in efficacy from the water extract (116.74 µg/mL) and acetone extract (117.64 µg/mL) (Table-1).

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