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Full Length Research Paper

Phenolic compounds and antioxidant activities of *Rumex hastatus* D. Don. leaves

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In this study different solvent fractions (n-hexane, ethyl acetate, chloroform, butanol and aqueous) of the methanol extract of *Rumex hastatus*(RH) leaves were evaluated for antioxidant activities. The ethyl acetate fraction possessed high amount of total polyphenolics (106.3±3.93 mg GAE/g extract) while butanolic fraction was found with high flavonoid contents (59.8±1.94 mg RTE/g extract) as compared to other solvent fractions. Butanol fraction exhibited most promising DPPH (EC_{50} 140±0.99 µg/ml), iron chelation (EC_{50} 70±2.1 µg/ml) and for phosphomolybdate (EC_{50} 31±1.25 µg/ml) scavenging activity. However, ethyl acetate fraction exhibited the highest scavenging potential for super oxide radicals (EC_{50} 40±0.78 µg/ml), ABTS radicals (EC_{50} 70±2.46 µg/ml), hydroxyl radicals (EC_{50} 9±0.56 µg/ml) and inhibition of β-carotene linoleic acid peroxidation (EC_{50} 54±5.67 µg/ml). Chloroform fraction showed the most potent antioxidant activities in scavenging of hydrogen peroxide (EC_{50} 48±4.89 µg/ml). The high correlation coefficient was found between EC_{50} values of DPPH, superoxides, hydroxyl, hydrogen peroxide and ABTS radicals with total polyphenolic and flavonoids. HPLC studies of ethyl acetate fraction indicated the presence of luteolin (24.67±2.90 mg/g extract), kaempherol (17.03±1.67 mg/g extract), luteolin-7-O-glucoside (14.73±2.17 mg/g extract) and rutin (8.24±1.43 mg/g extract). Therefore, our study substantiated that RH leaves can be used as a good source of potential antioxidant or functional food material due to the presence of sufficient amount of phenolics such as luteolin and kaempherol.

Key words: *Rumex hastatus,* antioxidant activity, flavonoids, phenolics, 2,2-diphenyl-1-picrylhydrazyl.

INTRODUCTION

Plants are well-known excellent perspectives for the discovery of new therapeutical products. The World Health Organization (WHO) estimates that 65 to 80% of the population of the developing countries depends on medicinal plants for basic pharmaceutical care (Shirwaikar et al., 2009). In recent years, an ample interest has been developed in finding natural anti-oxidants from commonly available green leafy vegetables and fruits, which though underexploited in most cases, possess a tremendous potential to overwhelm the deadly diseases of modern world. Numerous reports of crude extracts and pure natural compounds from plants have been appeared for antioxidant and radical-scavenging activities (Umamaheswari and Chatterjee, 2008; Kil et al., 2009). Within the antioxidant compounds, considerable attention has been devoted to flavonoids and phenolics. As plants are potential source of natural antioxidants and possess strong antioxidant activity and may help to protect cells against the oxidative damage caused by free radicals (Kahkonen, 1999). Due to the presence of the conjugated ring structures and hydroxyl groups, many phenolic compounds have the potential to function as antioxidants by scavenging or stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Shahidi and Wanasusdara, 1992). Therefore, the plant has promising compounds to be tested as potential antioxidant drugs for the remedy of diseases resulting from oxidative stress. The beneficial effects of intake of antioxidant substances have been shown in several experimental and epidemiological studies. In fact, literature has verified an association between intake of diets rich in fruits, vegetables, red wine with a decline in degenerative

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diseases (Ruch et al., 1989; Babu et al., 2001).

Rumex hastatus belongs to the Polygonaceae family and is popularly known as "khatimal". The plant is distributed in northern Pakistan, north east Afghanistan and south west of China, growing between 700 to 2500 m, sometimes grows as pure population (Qaiser, 2001). Leaves have a pleasant acidic taste and used in chutneys and pickles (Manan et al., 2007). It was reported that the whole plant of RH is used as medicine. It is laxative, alterative, tonic, used in rheumatism (Shinwari and Gilani, 2003) skin diseases, bilious complaints, piles, bleeding of lungs (Gorsi et al., 2002). The juice of plant is used for blood pressure (Manan et al., 2007). Literature demonstrates that RH is reported to possess activity or used in traditional systems of medicine for prevention and treatment of sexually transmitted diseases including AIDS (Vermani and Garg, 2001).

Two new phenolic compounds, hastatusides A and B together with five known compounds, resveratrol, rumexoside, torachrysone-8-yl β-D-glucopyranoside, rutin, nepodin, and orientaloside from the roots of *R. hastatus* has been reported (Zhang et al., 2009). In spite of the popular use, there is no report about secondary metabolites and antioxidant potential of RH leaves. This feature, allied to the importance of the oxidative stress in the pathogenesis of various diseases, prompted us to better evaluate the potential antioxidant properties of this plant.

The main objectives of this study were to determine the total phenolic and flavonoid contents and antioxidant capacity of leaves.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, aluminum chloride, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Ferric chloride ($FeCl_3$), Tween 80, β-carotene, (+)-catechin, Potassium persulphate, Folin-Ciocalteu's phenol reagent, ferrozine, gallic acid, rutin, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Sigma Co. (St. Louis, MO, USA). Sulphuric acid, deoxyribose, riboflavin, sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), disodium hydrogen phosphate (Na_2HPO_4) and hydrogen peroxide (H_2O_2) were obtained from Wako Co. (Osaka, Japan). Ferrous chloride ($FeCl_2$), potassium ferricyanide ($K_3Fe(CN)_6$), acetonitrile, triflouroacetic acid, sodium dihydrogen phosphate (NaH_2PO_4) and all solvents used were of analytical grade were purchased from Merck Co. (Darmstadt, Germany). Distilled deionized water (dd. H_2O) was prepared by Ultrapure TM water purification system (Lotun Co., Ltd., Taipei, Taiwan).

Plant collection

The plant was collected in March 2009 from Northern areas of Pakistan and the plant was identified by its vernacular name and later validated by Dr. Mir Ajab Khan, Department of Plant Sciences, Quaid-i-Azam University, Islamabad. A voucher specimen was deposited at the Herbarium of Pakistan Museum of Natural History, Islamabad.

Extract preparation

The leaves of the plant were dried under shade. The dried samples were powdered in a Willy Mill to 60-mesh size and used for solvent extraction. For sample preparation, 500 g of dried sample were extracted twice (2 x 2000 ml) 95% methanol at 25°C for 48 h and concentrated using a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) under reduced pressure at 40°C. The filtrate was suspended in water (50 ml) and n-hexane, ethyl acetate, chloroform and butanol (2 x 100 ml) was successively added, shake well and the layers were allowed to separate for 6 h in a separating funnel and at the last water soluble fraction was obtained. Each of the fractions obtained were dried using a rotary evaporator (Kil et al., 2009). The dry extract obtained with each solvent was weighed and expressed in terms of percentage of air dried weight of plant material.

Determination of total polyphenolic contents

The total polyphenolic contents were determined by the spectrophotometric method (Kim et al., 2003). In brief, a 1 ml portion of appropriately diluted extracts was added to 9 ml of deionized distilled water. Deionized distilled water was used as blank. One ml of Folin-Ciocalteu's phenol reagent was added to the mixture and then shaken. After 5 min, 10 ml of a 7% Na_2CO_3 solution was added with mixing. The mixed solution was then immediately diluted to volume (25 ml) with deionized distilled water and mixed thoroughly. After 90 min at 23°C, the absorbance was read at 750 nm. The standard curve for total polyphenolics was made using gallic acid standard solution (0 to 100 mg/l) under the same procedure as earlier described. The total polyphenolics were expressed as milligrams of gallic acid equivalents (GAE) per g of dried sample.

Determination of flavonoid contents

Total flavonoid content was determined by following Park et al. (2008). In a 10 ml test tube, 0.3 ml of extracts, 3.4 ml of 30% methanol, 0.15 ml of 0.5 M $NaNO_2$ and 0.15 ml of 0.3 M $AlCl_3.6H_2O$ were added and mixed. After 5 min, 1 ml of 1 M NaOH was added. The mixture was measured at 506 nm. The standard curve for total flavonoids was made using rutin standard solution (0 to 100 mg/l) under the same procedure as earlier described. The total flavonoids were expressed as milligrams of rutin equivalents per g of dried sample.

Antioxidant assays

Each sample was dissolved in 95% methanol at a concentration of 1 mg/ml and then diluted to prepare the series concentrations for antioxidant assays. Reference chemicals were used for comparison in all assays.

DPPH radical scavenging activity assay

The DPPH assay was done according to the method of Brand-Williams et al. (1995) with some modifications. The stock solution

was prepared by dissolving 24 mg DPPH with 100 ml methanol and then stored at 20°C until needed. The working solution was obtained by diluting DPPH solution with methanol to obtain an absorbance of about 0.980 (± 0.02) at 517 nm using the spectrophotometer. A 3 ml aliquot of this solution was mixed with 100 µl of the samples at varying concentrations (25 to 250 µg/ml). The solution in the test tubes were shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

Scavenging effect (%) = [(control absorbance-sample absorbance)/(control absorbance)] × 100.

EC_{50} value is the effective concentration that could scavenge 50% of the DPPH radicals. Ascorbic acid and rutin standard were used as positive references.

Superoxide anion radical scavenging assay

The assay for superoxide anion radical scavenging activity was based on a riboflavin-light-NBT system (Beauchamp and Fridovich, 1971). The reaction mixture contained 0.5 ml of phosphate buffer (50 mM, pH 7.6), 0.3 ml riboflavin (50 mM), 0.25 ml PMS (20 mM), and 0.1 ml NBT (0.5 mM), prior to the addition of 1 ml sample solution at varying concentrations (25 to 250 µg/ml). Reaction was started by illuminating the reaction mixture with different concentrations of the methanolic extract using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. Ascorbic acid was used as standard. The percent inhibition of superoxide anion generation was calculated using the following formula:

Scavenging activity (%) = (1- absorbance of sample/absorbance of control) × 100

Phosphomolybdate assay (total antioxidant assay)

The antioxidant activity of samples was evaluated by the phosphomolybdenum method according to the procedure of Umamaheswari and Chatterjee (2008). An aliquot of 0.1 ml of sample solution was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were capped with silver foil and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. Ascorbic acid was used as standard. The antioxidant capacity was estimated using following formula:

Antioxidant effect (%) = [(control absorbance-sample absorbance)/(control absorbance)] × 100.

Hydroxylradical scavenging assay

Hydroxyl radical scavenging activity of extracts was assayed by the method of Halliwell and Gutteridge (1981). The reaction mixture contained 500 ml of 2-deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.4), 200 ml of premixed ferric chloride (100 mM) and EDTA (100 mM) solution (1:1; v/v), 100 ml of H_2O_2 (200 mM) without or with the extract solution (100 ml). The reaction was triggered by adding 100 ml of 300 mM ascorbate and incubated for 1 h at 37°C. A solution of TBA in 1 ml (1%; w/v) of 50 mM NaOH and 1 ml of 2.8% (w/v; aqueous solution) TCA was added. The mixture was heated for 15 min on a boiling water bath and then cooled. The absorbance was measured at 532 nm. The scavenging activity on hydroxyl radical was calculated as follows:

Scavenging activity (%) = (1- absorbance of sample / absorbance of control) × 100.

Hydrogen peroxide-scavenging activity

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (1989). A solution of hydrogen peroxide (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Aliquots (0.1 ml) extracts sample was transferred into the test tubes and their volumes were made up to 0.4 ml with 50 mM phosphate buffer (pH 7.4). After addition of 0.6 ml hydrogen peroxide solution, tubes were vortexed and absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank. The abilities to scavenge the hydrogen peroxide were calculated using the following equation:

Hydrogen peroxide scavenging activity = (1- absorbance of sample/absorbance of control) × 100.

ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was performed by a slight modification of Re et al. (1999). Briefly, ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept for overnight in the dark to yield a dark colored solution containing ABTS radical cations. Prior to use in the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.700 (±0.02) at 745 nm, with temperature control set at 30°C. Free radical scavenging activity was assessed by mixing 300 µl of test sample with 3.0 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly one minute after mixing the solution, then up to 6 min. The percentage inhibition was calculated according to the formula:

Scavenging effect (%) = [(control absorbance-sample absorbance) / (control absorbance)] × 100.

The antioxidant capacity of test samples was expressed as EC_{50}, the concentration necessary for 50% reduction of ABTS.

β-Carotene bleaching assay

The assay was performed as given by Elzaawely et al. (2007) and modified slightly. First, 2 mg of β-carotene dissolved in 10 ml of chloroform was mixed with 20 mg of linoleic acid and 200 mg of Tween80 followed by chloroform removing under nitrogen and 50 ml of distilled water adding with vigorous shacking to prepare β-carotene linoleate emulsion. An aliquot of each sample (30 µl) was mixed with 250 µl of the emulsion, and then the absorbance was determined at 470 nm at 45°C for 2 h. β-Carotene bleaching inhibition was estimated as the following equation:

Bleaching inhibition (%) = (β-carotene content after 2 h of assay/ initial β-carotene content) × 100.

EC_{50} value is the sample concentration that could give 50% antioxidant ability. (+)-Catechin standard was used as a positive reference.

Table 1. Total phenolics, flavonoid and extraction yield of methanol extract and soluble fractions of RH leaves.

Plant extracts	Total phenolics (mg GAE/g of extract)	Total flavonoids (mg RTE/g of extract)	Extraction yield (%)
Methanol extract	89.1±3.71 b	47.57±1.27 b	9.5±1.27 a
n-Hexane fraction	53.8±2.63 d	6.4±1.13 d	3.7±0.61 d
Ethyl acetate fraction	106.3±3.93 a	45.72±1.76 b	5.32±0.28 c
Chloroform fraction	87.7±2.77 b	38.7±1.83 c	2.81±0.73 e
Butanol fraction	72.2±3.23 c	59.8±1.94 a	5.92±0.91 c
Aqueous fraction	46.4±2.78 d	6.54±1.84 d	7.7±1.69 b

Each value in the table is represented as mean ± SD (n = 3). Values in the same column followed by a different letter are significantly different ($p< 0.05$).

Iron chelating power

The ability of the extract to chelate iron (II) was estimated according to the method of Dastmalchi et al. (2008). The extracts were dissolved with methanol to prepare various sample solutions at 8, 6, 4, 2, 1 mg/ml and 500, 250 µg/ml. An aliquot of each sample (200 µl) was mixed with 100 µl of $FeCl_2.2H_2O$ (2.0 mM/l) and 900 µl of MeOH. After 5 min incubation, the reaction was initiated by the addition of 400 µl of ferrozine (5.0 mM/l). After 10 min incubation, the absorbance at 562 nm was recorded. The chelating activity (%) was calculated as the following equation:

Chelating activity (%) = [(control absorbance-sample absorbance) / (control absorbance)] × 100.

EC_{50} value is the effective concentration that could chelate 50% of iron (II). Catechin was used as controls.

Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). Extract solution (2 ml), phosphate buffer (2 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2 ml, 10 mg/ml) were mixed, and then incubated at 50°C for 20 min. Trichloroacetic acid (2 ml, 100 mg/l) was added to the mixture. A volume of 2 ml from each of the mixtures earlier mentioned was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) ferric chloride in a test tube. After 10 min reaction, the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated a high reducing power.

Quantification of flavonoids and phenolic compounds by HPLC: Phenolic compounds were measured at 280 nm using an Agilent HPLC (1100 series, Agilent Co., U.S.A). Separation was carried out through ZORBAX Eclipse, XDB-C18 column (5 µm; 4.6 × 150 mm, Agilent USA) with UV-VIS Spectra-Focus detector, injector-auto sampler. The mobile phase consisted of water with 0.05% trifluoroacetic acid (A) and (0.038% trifluoroacetic acid in 83% acetonitrile v/v) with the following gradient: 0 to 5 min, 15% B in A, 5 to 10 min, 70% B in A, 10 to 15 min, 70% B in A, at a flow rate of 1.0 ml/min and the injection volume was 10 µl. Compounds were detected by monitoring the elution at 280 nm. Identification of the phenolic compounds was carried out by comparing their retention times to those of standards. Content of phenolic compounds was expressed in mg/g extract.

Statistical analysis: All assays were carried out in triplicates and results are expressed as mean±SD. Statistical comparisons were done with one way ANOVA test. Differences were considered to be significant at $p<0.05$ or $p<0.01$. The EC_{50} values were calculated using the Graph Pad Prism 5 software.

RESULTS AND DISCUSSION

Extraction yield, total polyphenolics and flavonoid contents

The extraction yield of different fractions of RH leaves varied from 3.7±0.61 to 9.5±1.27% with a descending order of methanol > aqueous > butanol > ethyl acetate > n-hexane > chloroform fraction (Table 1). So the extractions with methanol resulted in the highest amount of total extractable compounds, whereas the extraction yield with chloroform was only small in comparison with that of the other solvents. Previous reports suggest that polyphenolic compounds are very important plant constituents, known to be good natural antioxidants. However, the activity of synthetic antioxidants was often observed to be higher than that of natural antioxidants. Table 1 summarizes that total phenolic compounds in fractions varied widely, ranging from 46.4±2.78 mg and 106.3±3.93 mg/g expressed as gallic acid equivalents (GAE). The methanolic extract exhibited the highest total phenolics content, whereas the contents obtained with n-hexane were much smaller that is in agreement with other reports (Ao et al., 2008). The content of flavonoids expressed as rutin equivalents, varied from 6.4±1.13 to 59.8±1.94 mg rutin equivalent/g extract. These amounts were comparable with results described in the literature for other extracts of plant products (Ao et al., 2008; Manian et al., 2008). It is well-known that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when ingested daily from a diet rich in fruits and vegetables. It may be due to the retard oxidative degradation of lipids to improve the quality and nutritional value of food.

Sharififar et al. (2009) reported that the rich-flavonoids plants could be a good source of antioxidants that would help to increase the overall antioxidant capacity of an organism and protect it against lipid peroxidation. So the result suggested that phenolic acids and flavonoids may

be the major contributors for the antioxidant activity to display characteristic inhibitory patterns toward the oxidative reaction *in vitro* and *in vivo*.

DPPH radical scavenging activity

DPPH is a stable free radical, antioxidant or radical scavenger changes its color from violet to yellow when is reduced by either the process of hydrogen- or electron donation (Brand-Williams et al., 1995). The DPPH radical has been widely used to assess the antioxidative activity of plant extracts and foods. It has been suggested that extracts which are rich in phenolics and flavonoids are involved in several biological activities including antioxidant ones. This indicates that the DPPH radical scavenging activities of all the fractions from RH leaves were related to the amount of antioxidant extracted by various solvents. Figure 1A shows that the scavenging effect of samples on DPPH radical was in the following order: butanol > methanol > ethyl acetate > chloroform > n-hexane and aqueous fractions. The EC_{50} values of scavenging DPPH radicals for the butanol and methanol fractions were 140±0.99 and 156±1.98 µg/ml, respectively, while for the n-hexane and aqueous fractions was > 500 µg/ml (Table 2). Though the antioxidant potential of fractions was less than those of ascorbic acid and rutin, the study revealed that butanol and methanol fractions have free radical scavengers or inhibitors, acting possibly as primary antioxidant.

In the present investigation, radical scavenging activity of extracts is thought to be related to the nature of phenolics, thus contributing to their electron transfer/hydrogen donating ability.

Superoxide radical scavenging activity

The superoxide anion is the most common free radical generated *in vivo*. Under oxidative stress, the concentration of this species can increase dramatically in all cells, inducing several pathophysiological processes, due to its transformation into more reactive species (Gülçin, 2007). Therefore, it was suggested to measure the comparative antioxidant ability of the extracts to scavenge the superoxide radical .The superoxide radical scavenging effect of different fractions of methanol extract of RH leaves was compared with the same doses of ascorbic acid ranging from 25 to 250 µg/ml as shown in Figure 1B. In fact, EC_{50} values in superoxide scavenging activities were in the order of ethyl acetate > methanol > chloroform > butanol > n-hexane and aqueous fraction (Table 2). All of the fractions had a scavenging activity on the superoxide radicals in a dose dependent manner. However, when compared to ascorbic acid, the superoxide scavenging activity of the extract was found to be low. In spite of this, ethyl acetate and methanolic fractions behave as strong superoxide anion quenchers. This could be due to the presence of bioactive compounds like phenolics and flavonoids in the extract which can scavenge superoxides and should be able to prevent oxidative damage of the major bio-molecules.

Phosphomolybdate assay

The phosphomolybdate method has been routinely used to assess the total antioxidant capacity of extracts (Prieto, 1999). In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green colored phosphomolybdenum V complex, which shows maximum absorbance at 700 nm. Figure 1C depicts the total antioxidant capacity of different fractions of methanol extract of RH leaves that can be ranked in the order of butanol > methanol > chloroform > ethyl acetate > n-hexane > aqueous fraction. The EC_{50} value of antioxidant capacity for the butanol and methanol fractions was 31±1.25 and 39±2.5 µg/ml, respectively, while for the aqueous fraction was 153±2.8 µg/ml (Table 2). This strong antioxidant activity of different fractions of RH leaves might be attributed to the presence of phytochemicals such as phenolic compounds. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants (Sharififar et al., 2009). The results obtained imply that the butanol and methanol fractions have notable antioxidant ability as compared to reference (ascorbic acid) antioxidant, seems to open an avenue for exploitation of cost effective natural antioxidants.

Hydroxyl radical scavenging activity

The hydroxyl radical is a highly reactive free radical, concerned as a detrimental species in pathophysiological processes and capable of damaging almost every molecule of biological system. The hydroxyl radical has the capacity to join the nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Babu et al., 2001). The highly reactive hydroxyl radicals can generate oxidative stress to biomolecules. The hydroxyl radical scavenging activity is shown in Figure 1D can be ranked as ethyl acetate > methanol > chloroform > n-hexane > butanol and aqueous fraction. All results showed antioxidant activity indose dependent manner at concentration 25 to 250 µg/ml. In the present investigation, the EC_{50} value of hydroxyl radical scavenging activity for the ethyl acetate and methanol fractions was 9±0.56 and 10±1.02 µg/ml while for the aqueous fraction was 20 ± 1.00 µg/ml (Table 2). The effect of different fractions of RH leaves on the

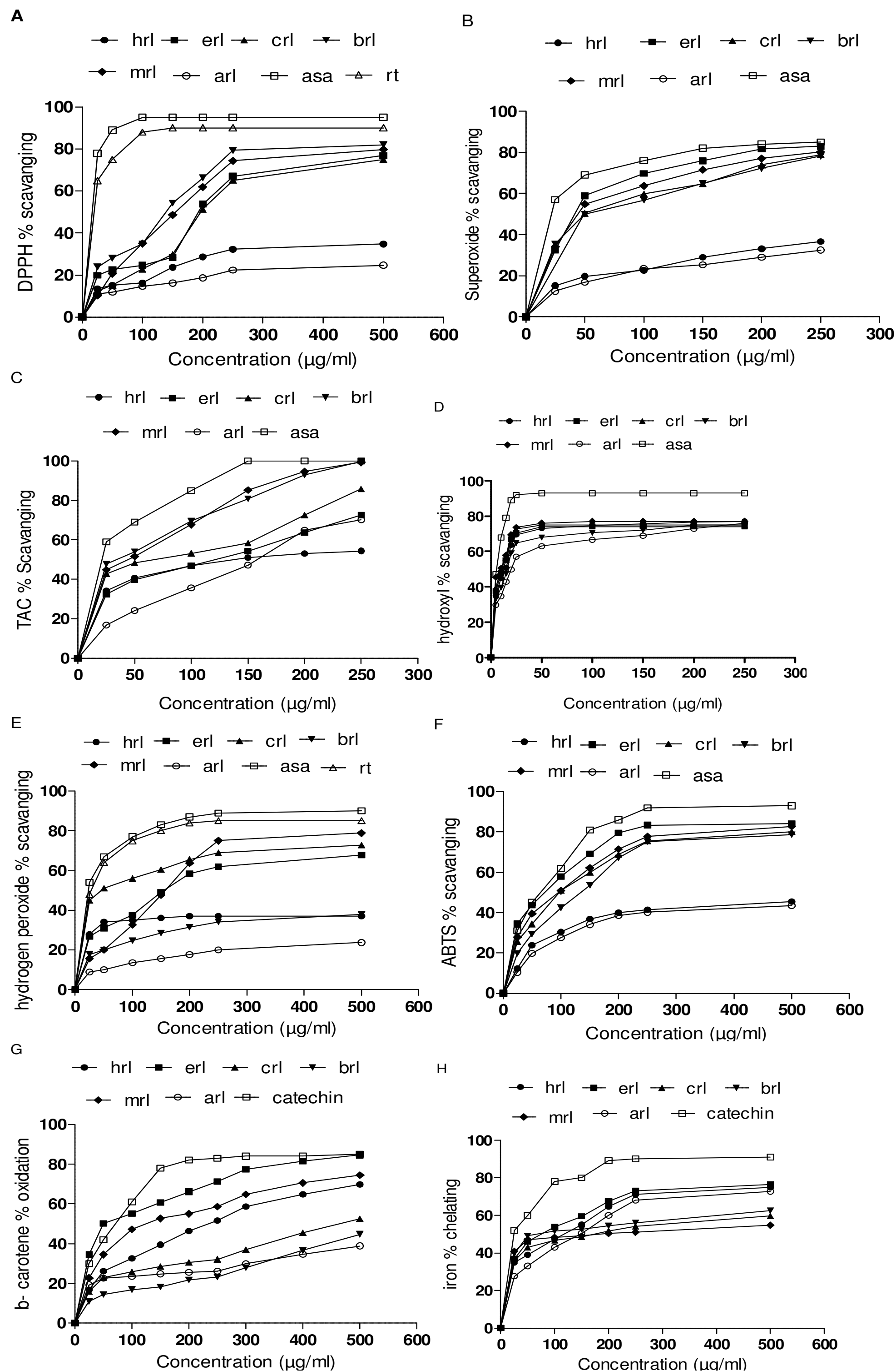

Figure 1. Antioxidant activities of different extracts from the methanol extract of RH leaves by different solvents at different concentrations. Each value represents a mean ± SD (n = 3): (A) DPPH radical scavenging activity, (B) Super oxide radical scavenging activity, (C) Total antioxidant capacity, (D)Hydroxyl radical scavenging activity, (E) Hydrogen peroxide radical scavenging activity, (F) ABTS radical scavenging activity, (G) β-carotene radical scavenging activity, and (H) Chelating power. hrl., n-hexane fraction of RH leaves, erl., ethyl acetate fraction of RH leaves, crl., chloroform fraction of RH leaves, brl., butanol fraction of RH leaves, mrl., methanol fraction of RH leaves, arl., aqueous fraction of RH leaves, asa., ascorbic acid, rt., rutin.

Table 2. Antioxidant effect (EC_{50}) on DPPH radicals, superoxide radicals, total antioxidant capacity and hydroxyl radicals of methanol extract and soluble fractions of RH leaves.

Plant extracts	EC_{50} µg/ml			
	Scavanging ability on DPPH radicals	Scavanging ability on superoxide radicals	Phosphomolybdate scavenging capacity	Scavanging ability on hydroxyl radicals
Methanol extract	156±1.98c	44±0.51b	39± 2.5e	10±1.02e
n-Hexane fraction	>500 a	>250a	136±1.67b	14±0.23c
Ethyl acetate fraction	194±1.34b	40±0.78b	113±2.2c	9±0.56e
Chloroform fraction	200±1.56b	49±0.45b	60±1.02d	12±0.61d
Butanol fraction	140±0.99c	53±0.66b	31±1.25e	17±0.92b
Aqueous fraction	>500a	>250a	153±2.8a	20±1.00a
Ascorbic acid	16±0.23d	21±0.37c	24±1.1e	6±0.67f
Rutin	22±0.56d	-	-	-

Each value in the table is represented as mean ± SD (n = 3). Values in the same column followed by a different letter are significantly different ($p< 0.05$). -, not determined.

prevention of free radical-mediated deoxyribose damage was evaluated. The Fentone reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Hydroxyl radicals may attack DNA either at the sugar or the base, generating a large number of products.

The ability of fractions to quench hydroxyl radicals might be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be attributed with good scavenger of active oxygen species, thus reducing the rate of chain reaction.

Hydrogen peroxide radical scavenging activity

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell (Halliwell, 1991). The scavenging effect of different fractions of RH leaves on hydrogen peroxide was concentration-dependent (25 to 500 µg/ml) as shown in Figure 1E. As compared with the EC_{50} values, the hydrogen peroxide scavenging activities of chloroform, ethyl acetate and methanol fractions was 48±4.89 µg/ml ,158±3.68 µg/ml and 158±4.97 µg/ml, respectively and more effective than that of aqueous and n-hexane > 500 µg/ml fractions (Table 3). The ability to scavenge hydrogen peroxide radicals various solvent extracts from RH leaves was in the order of chloroform > ethyl acetate > methanol > butanol > n-hexane and aqueous fraction. Similar to the results obtained from the ABTS, superoxide and hydroxyl scavenging assays, the chloroform, ethyl acetate and methanol fractions showed relatively strong hydrogen peroxide scavenging activity.

ABTS radical scavenging activity

Figure 1F shows that the ABTS radical scavenging ability of samples can be ranked as ethyl acetate > methanol > chloroform > butanol > n-hexane and aqueous fraction. The results obtained clearly imply that all the tested samples inhibit or scavenge the radical in a concentration dependent manner. The ethyl acetate, methanol and chloroform fractions from the leaves of RH exhibited the highest radical scavenging activities when reacted with the ABTS radicals. On contrary, the n-hexane and aqueous fractions did not show a leveling effect of at the highest concentration, however their radical scavenging effects were much less than rest of the fractions. It has been reported that the high molecular weight phenolics have more ability to scavenge free radicals (ABTS) and their effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups (Hagerman et al., 1998). Free radical (ABTS) scavenging activity of the leaves of RH might be due to the presence of high molecular phenolics in addition to the flavonoids. The EC_{50} values obtained for the ethyl acetate fraction (70±2.46 µg/ml) was significantly different ($p < 0.05$) from the EC_{50} values obtained for the n-hexane and aqueous (>500 µg/ml) fractions (Table 3).

β-carotene bleaching assay

The antioxidant activity with regard to the β-carotene bleaching assay of extract of RH leaves can be ranked as ethyl acetate > methanol > n-hexane > chloroform > butanol and aqueous fraction. β-carotene bleaching assay showed the dose response curve for all the fractions at concentrations ranging from 25 to 500 µg/ml (Figure 1G). The EC_{50} values of ethyl acetate and methanol fractions were 54±5.67µg/ml and 122±3.29 µg/ml, respectively (Table 3) which were comparable with catechin, β-carotene bleaching inhibition was reported in

Table 3. Antioxidant effect (EC_{50}) on hydrogen peroxide radicals, ABTS radicals, inhibition of β-carotene and iron chelating power of methanol extract and soluble fractions of RH leaves.

Plant Extracts	EC_{50} µg/ml			
	Scavanging ability on hydrogen peroxide radicals	Scavanging ability on ABTS radicals	β- carotene bleaching inhibition	Iron chelating power
Methanol extract	158±4.97b	98±1.42c	122±3.29c	184±2.81a
n-Hexane fraction	>500a	>500a	226±4.32b	112±2.13c
Ethyl acetate fraction	158±3.68b	70±2.46d	54±5.67e	72±1.03d
Chloroform fraction	48±4.89c	100±3.13c	464±4.82a	176±2.16a
Butanol fraction	>500a	128±4.51b	>500a	70±2.1d
Aqueous fraction	>500a	>500a	>500a	152±3.27b
Ascorbic acid	26±2.12d	60±1.32e	-	-
Catechin	-	-	74±2.29d	24±0.68e
Rutin	30±1.98d	-	-	-

Each value in the table is represented as mean ± SD (n = 3). Values in the same column followed by a different letter are significantly different ($p<0.05$). -, not determined.

various solvent extracts of dill (*Anethum graveolens*) flower (Shyu et al., 2009; Sahreen et al., 2010). The efficacy of extract of RH leaves to inhibit oxidation of linoleic acid emulsion is a indication of the complexity of the extract composition as well as potential, interaction between the extract and emulsion components. This data suggested that ethyl acetate and methanol fractions have a notable ability to react with free radicals to convert them into more stable non-reactive species and to terminate radical chain reaction.

Iron chelating activity

An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which possess the ability to catalyze hydro peroxide decomposition and Fenton-Type reactions. Chelating agents may act as secondary antioxidants because of their ability to reduce the redox potential to stabilize the oxidized form of the metal ions (Manian et al., 2008; Sahreen et al., 2010*)*. Therefore it was considered of importance to screen the iron (II) chelating ability of extracts. Figure 1H shows that all fractions were better ferrous ion chelators. The chelating activity was correlated well with the increasing concentration of each sample. The sequence for chelating power was butanol > ethyl acetate > n-hexane > aqueous > chloroform > methanol fraction. The iron chelating data measured at different concentrations (25 to 250 µg/ml) suggested that ferrous ion chelating effects of all the fractions of RH leaves would be rather beneficial to protect against oxidative damage. The EC_{50} value of iron chelating activity for the butanol and ethyl acetate fraction was 70±2.1 and 72±1.03 µg/ml while for the aqueous fraction was 184±2.81 µg/ml (Table 3). Accordingly it is suggested that the iron (II) chelating properties of these fractions may be credited to their endogenous chelating agents like phenolics and flavonoids. As some phenolic compounds have properly oriented functional groups, which can chelate metal ions to protect against oxidative damage.

Reducing power activity

In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{+3}/ferric cyanide complex to ferrous form by donating an electron. Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain (Gordon, 1990). Increasing absorbance at 700 nm indicates an increase in reducing ability. Figure 2 shows the dose-response curves for the reducing powers of all extracts (25 to 250 µg/ml) from RH leaves. It was found that the reducing power increased with concentration of each sample. The ranking order for reducing power was methanol > butanol > chloroform > aqueous > ethyl acetate > n-hexane fraction. The methanolic fraction from RH leaves exhibited a good reducing power of 1.332±0.14 at 250 µg/ml that might attributed to the collective antioxidant effects of phenolics and flavonoids.

Correlation with EC_{50} values of antioxidant activities and phytochemical contents

Through correlation analysis for phytochemical contents with EC_{50} values of radical scavenging activity of various soluble fractions of RH leaves and the contents of phenolics and flavonoids exhibited good correlation

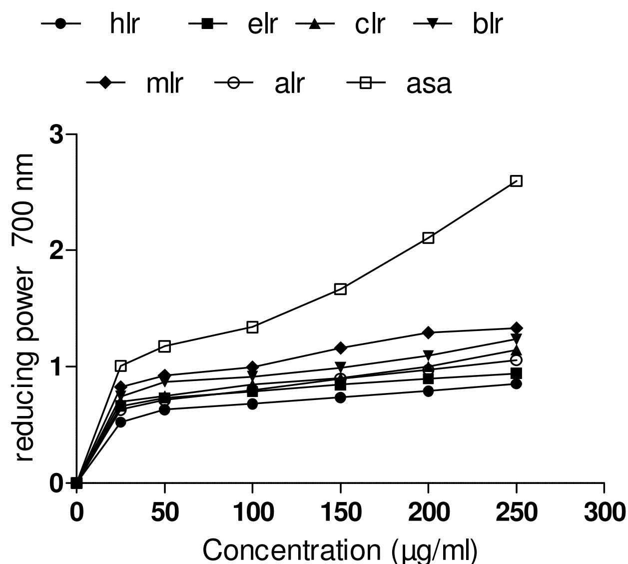

Figure 2. Reducing power of different extracts from the methanol extract of RH leaves by different solvents at different concentrations. Each value represents a mean ± SD (n = 3). hlr., n-hexane fraction of RH leaves, elr., ethyl acetate fraction of RH leaves, clr., chloroform fraction of RH leaves, blr., butanol fraction of RH leaves, mlr., methanol fraction of RH leaves, alr., aqueous fraction of RH leaves, asa., ascorbic acid.

Table 4. Correlations[1] between the EC_{50} values of antioxidant activities and phenolic and flavonoids content of RH leaves.

Assays	Correlation R^2	
	Phenolics	Flavonoids
EC_{50} of DPPH radical scavenging ability	0.6763*	0.9610***
EC_{50} of superoxide radical scavenging ability	0.7979*	0.8957**
EC_{50} of phosphomolybdate antioxidant capacity	0.2512	0.7536*
EC_{50} of hydroxyl radical scavenging ability	0.7439*	0.1944
EC_{50} of hydrogen peroxide radical scavenging ability	0.7024*	0.2008
EC_{50} of ABTS radical scavenging ability	0.8346*	0.8692**
EC_{50} of β-carotene bleaching inhibition	0.3127	0.01551
EC_{50} of iron chelating power	0.0086	0.05942*

[1]R. hastatus leaves methanolic extract and its soluble fractions were used in the correlation. *,**, *** shows significance at $p<0.05$, $p<0.01$ and $p<0.001$probability level.

($R^2 > 0.7159$) with DPPH, Superoxide and ABTS radical scavenging activities (Table 4). However, non significant correlation was found in case of iron chelating power and β-carotene bleaching inhibition. In addition, EC_{50} of phosphomolybdate assay, hydrogen peroxide and hydroxyl radical scavenging assays presented significant correlation with phenolics while non significant with flavonoids. The results indicate that phenolic acids and flavonoids are the major contributors to the antioxidant activities of fractions of RH leaves, and highlighted the importance of phenolic compounds in the antioxidant behaviour of plant extracts. The noticeable correlation among different tests showed that the antioxidant assays selected in the present investigation are viable and complementary to the antioxidant activities. Our results are in agreement with Ao et al. (2008) who reported strong relationship with DPPH and ABTS as compare to β-carotene.

Analysis of ethyl acetate fraction by HPLC

Since the ethyl acetate fraction exhibited the strongest

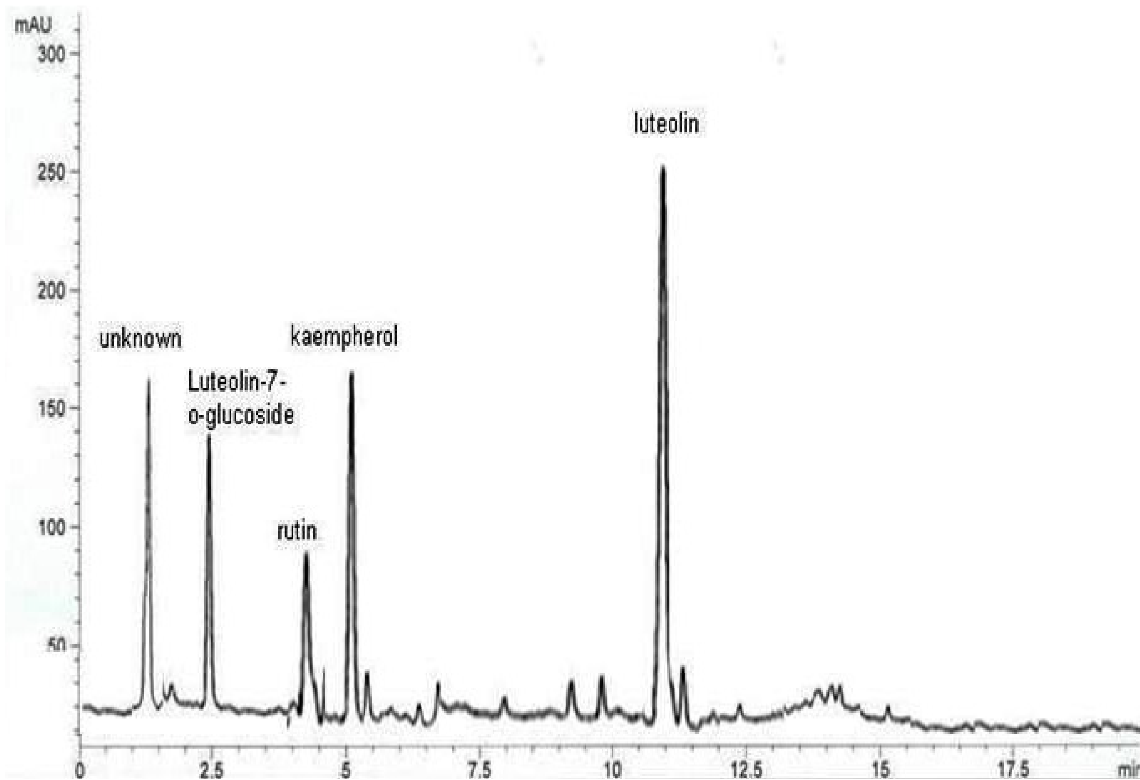

Figure 3. HPLC chromatogram of ethyl acetate fraction from the methanol extract of RH leaves.

Table 5. Phenolic compounds content of ethyl acetate fraction of RH leaves.

Phenolic compounds of ethyl acetate fraction	Content (mg/g of extract)
Luteolin	24.67±2.90
Kaempherol	17.03±1.67
Luteolin-7-O-glucoside	14.73±2.17
Rutin	8.24±1.43
Unknown	15.93±2.10

Each value in the table is represented as mean ± SD (n = 3).

antioxidant activity, it was analyzed by HPLC for the presence of phenolic compounds. The ethyl acetate fraction contained a variety of phenolic compounds (Figure 3). By comparing the retention time of these compounds with those of standards, four phenolic compounds were identified and one was unknown (Table 5). In addition, HPLC results indicate that in this fraction luteolin (24.67±2.90 mg/g) was the predominant compound, followed by kaempherol (17.03±1.67 mg/g). Flavonoids, and phenolic acids are important feedative antioxidants. The results suggest that the activity of the ethyl acetate fraction is attributed to these phenolic compounds and in particular to luteolin, kaempherol, luteolin-7-O-glucoside and rutin, however unknown compound may also involve in the antioxidant activities of RH leaves.

Conclusion

The present investigation suggests that RH leaves possess potential antioxidant activity, especially with reference to ethyl acetate and methanolic fractions. The activity of these fractions may be attributed to the presence of phenolic and flavonoid contents. Thus, our results suggested that the extract can be utilized as an effective and safe antioxidant source, although the antioxidant activities of ethyl acetate and methanolic

fractions were lower than that of reference compounds. It can be concluded that such identified potential and natural constituents could be exploited as cost effective food/feed additives for human and animal health. However, further isolation and characterization of bioactive compounds from the aforementioned *Rumex* samples, their impact on various health improvements and control of free radical mediated diseases through *in vivo* studies is needed.

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