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Total phenols, flavonoids, anthocyanins, ascorbic acid contents and antioxidant activity of *Rhamnus kurdica* Boiss for flower and leaves in flowering and pre-flowering stages

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The antioxidant capability, total phenol, total flavonoid, anthocyanins, ascorbic acid contents, and reducing power contents of polar and non-polar extracts for flower and leaves in two stages of growth for *Rhamnus kurdica* Boiss in flowering were evaluated in this work. The polar extraction of flower of *R. kurdica* Boiss had a higher total phenolic content as well as antioxidant activity compared to that of the other subfractions of flower and leaves in the different stages. Antioxidant activities of the samples were determined by three various testing systems namely 2, 2-diphenyl-1-picrylhydrazyl (DPPH), b-carotene/linoleic acid and reducing power assay. In DPPH system, the highest radical scavenging activity was seen by the polar subfraction in flowers of methanol extract [21.04±1.35 (µg/ml)]. Our findings demonstrate that the methanolic extracts of *R. kurdica* Boiss may be suggested as a new potential source of natural antioxidant.

Key word: *Rhamnus kurdica* Boiss, antioxidant, total phenolics, flavonoid, anthocyanins.

INTRODUCTION

Antioxidant activity is essential for life, to counteract the strongly oxidizing environment in which we live (Velioglu et al., 1998). Free radical formation is controlled naturally by compounds known as antioxidants. The damage in biological systems can be cumulative when the concentration of radical species and antioxidants are not in balance (Erkan et al., 2011). Phenolic compounds such as flavonoids, phenolic acids, diterpenes, and tannins have received attention for their high antioxidative activity (Rice-Evans et al., 1996).

Converging evidence from both experimental and epidemiological studies have demon-strated that cereals, vegetables, and fruits contain a myriad of phenolic compounds (Maheshwari et al., 2011). Natural antioxidants are compounds that increase the lifetime period and the nutrition value of food. They are transferred to beverages during the production process from different parts of plants (leaves, fruits, etc). Their beneficial effect on the health of consumers is seen mainly in reducing the concentration of free radicals and in decreasing hypertension (Theodoridis et al., 2011).

To the best of our knowledge, there is no information on the antioxidant properties of *R. kurdica* Boiss. The aim of this work was to evaluate the *in vitro* antioxidant properties of the methanol extracts of *R. kurdica* Boiss for flowers and leaves in two stages by DPPH, β-carotene/linoleic acid, reducing power assays, total phenolics, anthocyanins.

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MATERIALS AND METHODS

Plant material

The aerial parts of *R. kurdica* Boiss in different stage were gathered before flowering (pre-flowering) and flowering period in summer 2011 in the west of Iran. The aerial parts (leaves and flowers) were dried in shade (at room temperature). The plants were identified and authenticated by the Laboratory of Botanic Ecology of the Razi University. A voucher specimen was deposited at the chemistry herbarium of this laboratory under the code 2010 RKB.

Chemicals

Linoleic acid, 2, 6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95%), gallic acid, oxalic acid, ascorbic acid (AA), catechin, PVPP (polyvinylpolypyrrolidone), cyanidin-3-glucoside and β-carotene, were procured from Sigma-Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, and HPLC grade chloroform, standard Folin-Ciocalteu's phenol reagent, anhydrous sodium sulphate, ferric chloride, sodium carbonate, potassium ferricyanide, phosphate buffer solution (PBS), and Tween 40 were obtained from Merck (Darmstadt, Germany).

Preparation of the methanol extract

60 g of the dried leaves in different stage of growth and 20 of powdered flower of *R. kurdica* Boiss were extracted with methanol by using Soxhlet apparatus at 50°C for 18 h. The extract was filtered and concentrated under vacuum at 60°C by using a rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany), yielding a waxy material for leaves in per-flowering, leaves in flowering and flower were (3.73 g, 6.25%), (4.16 g, 6.93% w/w), and (1.08 g, 5.40%) respectively. These extracts were suspended in water and extracted with chloroform (4 ×100 ml) to obtain 2.11 g (3.51%), 2.97 g (4.95%), and 0.61 g (3.05%) polar and 1.22 g (2.03%), 1.04 (1.73%), and 0.38 g(1.9%) non-polar extracts. The extracts were stored in darkness at 4°C until used within a maximum period of one week.

Antioxidant properties

1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity

The free radical-scavenging activities of extract were measured by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) as described by Sharififar et al. (2007) with some modification. 3 ml of various concentrations of the extract was added to 1 ml of a 0.5 mM methanol solution of DPPH. The mixture was strongly shaken and left to stand at room temperature for 60 min in the dark. Then, the absorbance was measured at 517 nm against a blank. Determinations were made on a Shimadzu UV-visible spectrophotometer; model UV 160 U (Japan). Inhibition of free radical, DPPH, in percent (I %) was calculated according to the formula:

$$I\% = ((A_b \_ A_S)/A_b) \times 100$$

Where, A_b is the absorbance of the control reaction (containing all reagents except the test compound), and As is the absorbance of the test compound.

The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against sample concentration. Tests were carried out in triplicate. Ascorbic acid (AA) was used as positive control.

β -Carotene linoleic acid assay

The antioxidant activity was evaluated according to the method described by Miller (1971) with some modifications. Briefly, 1.5 ml of β-carotene solution (1 mg/ml in chloroform), 3 ml of linoleic acid solution (10 mg/ml in chloroform), and 1.0 ml of Tween 40 solution (300 mg/ml in chloroform) were pipetted into a 250 ml flask. The chloroform was removed by rotary vacuum evaporator, and 150 ml deionized water was added to the residue and the mixture was shaken to form an emulsion. Three hundred and fifty microliter (350 µl) of test sample in methanol (2 mg/ml) was mixed with 2.5 ml of this reagent, and the emulsion system was incubated for up to 24 h at room temperature. The same procedure was repeated with the synthetic antioxidant, BHT as positive control, and a blank containing only 350 µl of methanol. After this incubation period, absorbances of the mixtures were measured at 490 nm. Antioxidative capacities of the extract were compared with those of BHT and blank.

Reducing power

The reducing power of extract was determined according to the method of Sfahlan et al. (2009) with some modifications. Different concentrations of methanolic extract (polar and nonpolar) of plant in methanol (1.0 ml) were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 *g* for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. Ascorbic acid was used as positive control.

Determination of total phenolic contents

Total phenolic contents of the extract and the oil were determined using the Folin-Ciocalteu reagent according to the method of Singleton and Rossi (1965) using gallic acid as standard, with some modifications. The extract solution (0.1 ml) containing 1000 µg of the extract was mixed with 46 ml of distilled water in a volumetric flask and 1 ml Folin-Ciocalteu reagent was added, and the flask was thoroughly shaken. The mixture was allowed to react for 3 min and 3 ml aqueous solution of 2% Na_2CO_3 was added. At the end of incubation of 2 h at room temperature, absorbance of each mixture was measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. Total phenol contents were expressed as µg gallic acid equivalents per mg of the extract. All tests were carried out in triplicate, and gallic acid equivalent values were reported as X ± SD of triplicates.

Determination of total flavonoids

A modified protocol of that described by Kim et al. (2003) was employed. A 0.1 ml aliquot of methanolic extract, appropriately diluted, was mixed with 0.4 ml distilled water in a 1.5 mL microcentrifuge tube, 0.03 ml of 5% $NaNO_2$ was added and the mixture was allowed to react for 5 min. Following this, 0.03 ml of 10% $AlCl_3$ was added and the mixture stood for a further 5 min. Finally, the reaction mixture was treated with 0.2 ml of 1 M Na_2CO_3 and 0.24 ml distilled water, and the absorbance at 510 nm was obtained against a blank prepared similarly, by replacing extract with distilled water. Total flavonoid content was calculated from a calibration curve using catechin as standard, and expressed as mg catechin equivalents (CTE) per 100 g plant (Dourtoglou et al.,

Table 1. Antioxidant activities and total phenol of *Rhamnus kurdica Boiss* methanol extract in two stage of growth for leaves and flowers.

Sample	DPPH IC_{50} (µg/ml)	β-Carotene bleaching (RAA) (%)	Total phenol contents (µg/mg)
Polar subfraction $^{L-P}$	47.16±3.83	78.84±1.96	212.72± 8.82
Nonpolar subfraction $^{L-P}$	321.49±9.61	34.29±1.43	49.87±2.87
Polar subfraction $^{L-F}$	31.11±3.57	98.06±3.98	252.33±7.18
Nonpolar subfraction $^{L-F}$	287.02±7.47	54.77±1.37	76.30±3.47
Polar subfraction^{F}	21.04±1.35	99.13±3.12	307.38±10.34
Nonpolar subfraction^{F}	98.36±4.02	61.39±0.67	102.51±4.21
BHT	19.5 ± 1.06	100	nd
Ascorbic acid	5.1 ± 0.87	nd	nd

$^{L-P}$Leaves in pre - flowering; $^{L-F}$ leaves in flowering; ^{F}flower. Nd, Not detected.

2006).

Anthocyanins

Total anthocyanin content was measured with the pH differential absorbance method, as described by Cheng and Breen (1991). Briefly, absorbance of the extracts were measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetate acid- sodium acetate, 1 M). Anthocyanin content was calculated using a molar extinction coefficient of 29,600 (cyanidin-3- glucoside) and absorbance of

$$A = [(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}]$$

Results were expressed as µg cyanidin-3-glucoside equivalents 100 mg^{-1} fw (Pantelidis et al., 2007).

Ascorbic acid content

Ascorbic acid of the extracts was determined using ascorbic acid as standard, with some modifications. The samples (1 g) and 4 ml oxalic acid (1%) were mixed, homogenised for 1 min, and filtered. Polyvinylpolypyrrolidone (PVPP) (100 g) was added to 2.5 ml of the filtered sample, to remove phenols, and 2-3 drops of H_2SO_4 (25%) were added, to reduce the pH to below 1. Absorbance of the mixture was determined at 254 nm. Results were expressed as mg ascorbic acid (AA) 100 g^{-1} fresh weight (fw) (Pantelidis et al., 2007).

RESULTS AND DISCUSSION

Antioxidant properties

Free radical-scavenging activity

The free radical-scavenging abilities of the methanol extract of *R. kurdica* Boiss and polar and nonpolar fractions (water, chloroform) against DPPH were tested, and the results are presented in Table 1. The polar subfraction for flower of methanol extract provided the highest radical-scavenging activity with the lowest IC_{50} value of 21.04±0.35µg/ml than the other subfractions. The antioxidant activities of the plant extract was also evaluated by the spectrophotometric β-carotene bleaching test. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (Kulisic et al., 2004). The relative antioxidative activities (RAAs) of the extracts were calculated from the equation, RAA = A sample/A BHT, where A BHT is the absorbance of the control (BHT) and A sample is the absorbance of the extract. The calculated RAAs of the extract are given in Table 1. In the reducing power assay, the presence of antioxidants in the sample would result in the reducing of Fe^{3+}-Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue $Fe^{4+}[Fe(CN)6]^{3+}$ at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1a, b, c shows the reducing power of the methanolic (polar and non-polar) extract of *R. kurdica* Boiss as a function of their concentrations. The amounts of total phenolics in the extract for different stage were determined spectrometrically according to the Folin-Ciocalteu procedure and calculated as gallic acid equivalents. Gallic acid is a water-soluble polyhydroxyphenolic compound that can be found in various natural plants. The standard curve equation was, y (absorbance) = 0.0003 ×gallic acid (µg) + 0.00534. The amounts of total phenols found in the plant methanolic extract are shown in Table 1.

Flavonoid

The standard curve equation for determination flavonoids with catechin is y (absorbance) = 0.004× catechin (µg) + 0.0483. The amounts of total flavonoids found in plant methanolic extract for flowers of *R. kurdica* Boiss was 101.17 ± 5.74 µg/mg, for leaves in flowering stage was 86.32 ± 2.98 µg/mg and in pre-flowering stage for leaves was 51.67 ± 1.54 µg/mg.

Anthocyanins

Significant differences in anthocyanin content were

Table 2. Flavonoid, anthocyanins and ascorbic acid contents of *R.kurdica Boiss* methanol extract in two stages of growth for leaves and flowers.

Sample	Flavonoid µg/mg	Anthocyanin (µg 100 mg^{-1} fw)	Ascorbic acid (µg 100 mg^{-1} fw)
Flower	101.17± 5.74	21.53±0.57	25.13±1.59
Leaves in flowering	86.32±2.98	12.36±0.84	16.87±1.02
Leaves in pre - flowering	51.67±1.54	2.27±0.03	14.06±1.07

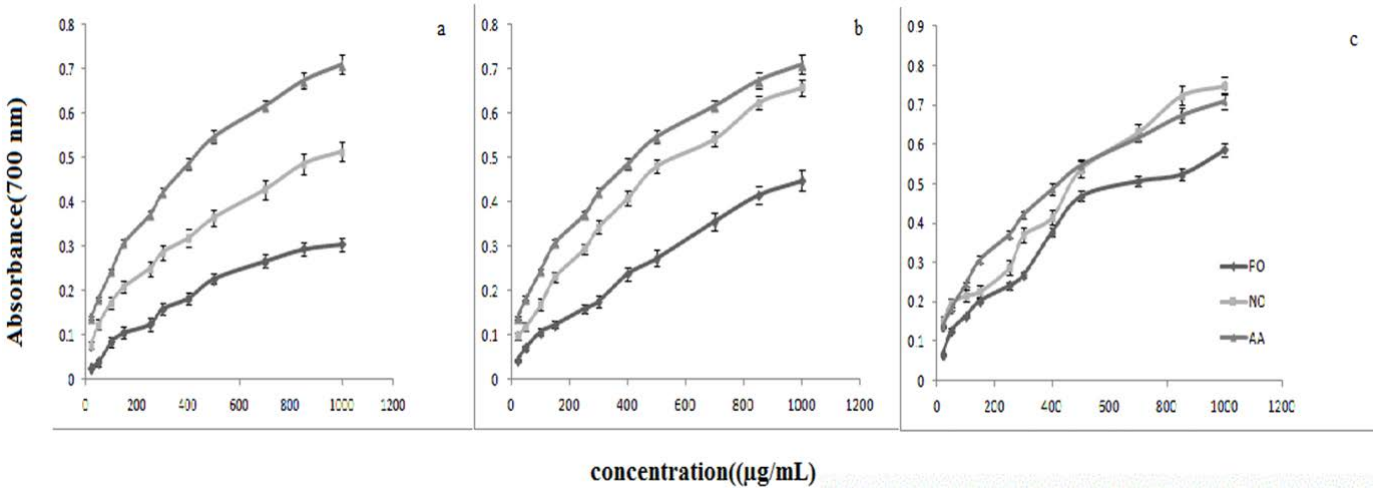

Figure 1. Reducing power of different concentrations of methanolic extract polar and non-polar subfraction. a: leaves in the pre-flowering stage, b: leaves in the flowering stage and c: flowers of *Rhamnus kurdica Boiss* compared to ascorbic acid, (spectrophotometric detection of the Fe^{+3}-Fe^{+2} transformations). Polar (PO) and non-polar (NO) subfraction extract; AA, ascorbic acid.

recorded, since these pigments are responsible for the red and blue color. The nonpolar subfraction in flowering stage contained the highest anthocyanin content expressed as cyanidin-3- glucoside. The results are shown in Table 2. The results show relation between anthocyanins and antioxidant activity.

Ascorbic acid

Significant differences in ascorbic acid content among the different sub-fraction are recorded in Table 2. The flowers subfraction had the highest content of ascorbic acid (25.13 ± 1.59 mg 100 g^{-1} fw).

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