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Total Phenolic Content & Antioxidant Activity of Roselle (*Hibiscus Sabdariffa* L.) Calyces Extracts.

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ABSTRACT

Total phenolic content and antioxidant activity were determined in Roselle (*Hibiscus Sabdariffa* L.) calyces extracts. Dried Roselle calyces were collected from Fenoj Region in Sistan & Baluchestan province of Iran. Liquid extracts of dried Roselle (*Hibiscus Sabdariffa* L.) calyces obtained using water, ethanol (50%), methanol (50%), ethanol-methanol (50-50%, v/v) to evaluate selected phenolic compounds and antioxidant activity using the β -carotene bleaching method, free-radical scavenging by DPPH. The phenolic compounds contents ranged from 24.36 (in water) to 44.43 (in ethanol-methanol) mg of gallic acid 100 g⁻¹ of dried calyces, and the antioxidant activity ranged from 39.45 % (in water) to 60.43 % (in ethanol-methanol). Free radical scavenging capacity of extracts by DPPH were obtained in range of 43.85% (in water) to 91.77% (in ethanol-methanol). The antioxidant properties of Roselle calyces make the extracts ideal for use in foods as a natural extract, concentrate, or powder.

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INTRODUCTION

Hibiscus sabdariffa L., commonly known as roselle or red sorrel, is a tropical plant which belongs to the family Malvaceae, widely grown in central and West Africa and South-East Asia. In the early 1970s roselle has expected great considerable attention as a potential source of natural food colorant, pharmaceutical and cosmetics. The thick, red and fleshy, cup shaped calyces of the roselle flowers are consumed worldwide as food or a food ingredient such as jellies, syrups, beverages, puddings, cakes, wines and as a colorant. In addition to their use in food, various parts of the roselle plant have been used in traditional medicine for the prevention of disease such as cardiovascular disease, liver disease, fever and hypertension. Previous studies have shown that roselle can prevent cancer, lower blood pressure and improve the digestive system in humans. Its calyces extract has also been used as an effective treatment for patients with kidney stones (Mazza and Miniati, 1993).

Roselle extracts can also perform as an antioxidant. For example, it protects against low density lipoprotein (LDL)-oxidation and has hypolipidemic effects in vivo (Hirupanich *et al.*, 2006).

Roselle is an important source of vitamins, minerals, and bioactive compounds, such as organic acids and polyphenols, some of them with antioxidant properties.

The most essential part in roselle is calyces. Roselle calyces are potentially good source of phenolic compounds (Al-Hashimi, 2012) and anthocyanins (Prenesti *et al.*, 2007). These compounds could be a major determinant of antioxidant potentials of foods, and a natural source of antioxidants. The research on phenolic compounds has been growing lately because of the increasing worldwide demand for phenolic compounds and their increasing application in food industry (Rodrigues and Pinto, 2007).

Extraction is the first step in the isolation of phenolic compounds from plant materials such traditional methods as maceration and soxhlet extractions, which have been used for many decades.

An extraction technique for roselle calyces extraction plays a major role in an antioxidant activity of the extract. Water extraction of calyx has considerable economic potential and produces a brilliant red color extract, rich in anthocyanins and hibiscus acid (Al-Kahtani, 1990).

Mazza and Miniati (1993) reported that roselle anthocyanin extract produced by using water at 50°C for 4 hours with a calyx/solvent ratio of 1:10 behaved as first-order kinetics of thermal degradation which was rapid

at temperatures above 100°C, and virtually instantaneous at 165°C to 170°C. Currently, only a few studies have been reported (Tsai *et al.*, 2002) with regard to the effects of processing techniques, such as extraction and concentration on the properties especially antioxidant activity of roselle extract.

The aim of this study was to evaluate the antioxidative activity of aqueous and alcoholic extracts of calyx, and to evaluate the relationship between the antioxidative activity and total phenolic content of the roselle.

MATERIAL AND METHODS

1.1. Plant material:

Dehydrated roselle calyces, dried at 30±2 °C using solar drying, were obtained from Fenooj region in Sistan & Baluchestan province of Iran and maintained in aluminum bags under refrigerated (3±1) °C until used for the analysis. Before extraction process, the roselle calyces were ground using a household blender. Moisture, protein, fat, ash and fiber were determined according to methods described in the AOAC (2000). To evaluate the pH, 5 g of Roselle powder was mixed with 5 milliliters of distilled water, stirred for 30 minutes, and filtered through Whatman paper No. 1. The pH was measured using a Jemway 3310 pH meter (Staffordshire, UK). The total soluble solids (°Brix) were measured using a hand-held refractometer (Atago Co. LTD, Tokyo, Japan). The total acidity content of the extract as malic acid (%) was determined according to the method of AOAC (2000).

1.2. Chemicals:

1, 1-diphenyl-2-picrylhydrazyl (DPPH), β -carotene, tween-20, linoleic acid, and Gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteu reagent, phosphoric acid, acetonitrile, Ethanol and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany), sodium bicarbonate and chloroform were purchased from BDH Chemicals (Poole, England).

1.3. Extraction:

Roselle calyces extracts were obtained according to the Ersus and Yurdagel (2006) method, with modifications. Water, methanol: water (50-50, v/v), ethanol: water (50-50, v/v) & methanol: ethanol (50-50, v/v) were used for the extractions. 10 g of dried and ground roselle calyces and 100 ml of each solvent were sealed in an erlenmayer flask, subjected to shaking at room temperature overnight at a speed of 1000 vib/min. After centrifugation; supernatant was filtered through a whatman paper No.1. The solvent was removed from extract under reduced pressure at 40 °C by using a rotary evaporator (Rotavapor R-200, Buchi Labortechnik, Switzerland) until sticky extract was obtained. It was then kept at refrigerator prior to further use. The yield of extract was measured by gravimetry.

1.4. Determination of total phenolic compounds (TPC):

The total phenolic content in the supernatant was determined using Folin–Ciocalteu method with some modifications (Martinez *et al.*, 2004). Briefly, 800 μ l of sample (1g L⁻¹) was mixed with 800 μ l of Folin–Ciocalteu reagent. After 5 min, 800 μ l of sodium bicarbonate (0.01 M) and 5 ml of distilled water were added to the mixture. The absorbance was measured at 760 nm using a UV–visible spectrophotometer (UV-1601; Shimadzu Corp., Kyoto, Japan).

The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of plant material using a regression equation and a Gallic acid calibration curve ($R^2=0.995$)

1.5. Determination of antioxidant activity:

The antioxidant activities of roselle extract were evaluated, using the β -carotene bleaching method described by Velioğlu *et al.*, (1998) with modifications. 1 ml of a 0.2 mg/ml β -carotene solution in chloroform was added to flasks containing 0.02 ml of linoleic acid and 0.2 ml of Tween-20. The chloroform was removed at 40 °C using a rotary evaporator (Rotavapor R-200, Buchi Labortechnik, Switzerland) for 5–10 min. The resultant mixture was immediately diluted with 100 ml of distilled water and mixed for 1–2 min to form an emulsion. A mixture prepared similarly but without β -carotene, was used as a blank. A control containing 0.2 ml of 80% (v/v) methanol instead of extract was also prepared. A 5 ml aliquot of the emulsion was added to a tube containing 0.2 ml of the sample extract at 1 mg/ml. BHT (1 mg/ml), in methanolic solution, was used as a standard. The tubes were placed in a water bath (Sb-16, Techne, England) at 40 °C for 2 h. Absorbance was read at 470 nm at 15 min intervals, using a UV–visible spectrophotometer (UV-1601; Shimadzu Corp., Kyoto, Japan). The antioxidant activity of each sample was calculated as percent inhibition relative to the control, using the following equation:

$$\% \text{ Antioxidant activity} = [1 - (A_0 - A_{t0}/A_0 - A_t)] \times 100$$

A_{t0} and A_0 are the absorbance values measured at zero time of incubation for sample extract and control, respectively.

A_t , and A_0^0 are the absorbance values for sample extract and control, respectively, at 120 minutes.

1.6. Measurement of DPPH radical-scavenging activity:

Free radical-scavenging was evaluated using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method of Kahkonen *et al.*, (1999). 1ml of freshly prepared 1 mM DPPH in methanol was added to test tubes containing 5 ml of the sample extracts. A control was prepared by adding 1 ml of DPPH solution to 5 ml of 80% methanol. Following storage in the dark for 30 min, the absorbance was read at 517 nm using a UV–visible spectrophotometer (UV-1601; Shimadzu Corp., Kyoto, Japan).

The percentage of free radical-scavenging activity was calculated, based on the following equation.

Free radical-scavenging activity % = $[1 - (A_1 / A_0)] \times 100$

A_1 = Absorbance of sample at 517 nm

A_0 = Absorbance of control at 517 nm

1.7. HPLC analysis:

Analysis was performed on a HPLC (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A UV detector and a SIL-20AC TH autosampler coupled with an analytical software (LC Solution-Release 1.23SP1) were applied. A 250 nm $\times$ 4.6mm i.d., 5 μ m, Eclipse XDB-C18 column (Agilent) column was used. The column thermostat was set at 30 °C to obtain the best resolutions and a stability of the retention time. Analytical HPLC was carried out according to the method described as follows: Mobile phase A consisted of 4% phosphoric acid; mobile phase B 100% acetonitrile. The Linear gradient was used from 10% to 100% acetonitrile in 30 min; with a flow rate of 1 ml/min. Injection volume was 15 μ L. Each extract was centrifuged, filtered through a 0.45 μ m nylon filter and analyzed three times directly by HPLC running. After each run, the column was equilibrated for 10 min under initial conditions (Carrera *et al.*, 2012).

1.8. Statistical analysis:

All chemical analyses performed on triplicate samples which were reported as mean $\pm$ standard deviations. ANOVA test was performed according to Mstat C and Excel software. The experimental design was a Complete Randomized Design (CRD). Significant differences between the means of parameters were determined by using the Duncan ($P \leq 0.05$).

RESULTS AND DISCUSSION

Table 1 shows the chemical analyses of dried roselle calyces. According to table 1, moisture content of naturally dried roselle calyces was 11.35%. Chemical analysis also showed that roselle calyces contained 6.42, 0.33 and 10.27 % of protein, fat and fiber, respectively. Results given in the same table indicated that roselle calyces had high ash content which reached 11.02 %. The composition of the roselle calyces was similar to referenced data with some differences that may be due to genetic variety and type of soil (Babalola *et al.*, 2001).

Table 1: Chemical composition of roselle (*Hibiscus sabdariffa* L.) calyces (on dry weight basis).

Test	Results
Moisture (%)	11.35 $\pm$ 0.01
Protein	6.42 $\pm$ 0.04
Fat	2.33 $\pm$ 0.06
Ash	11.02 $\pm$ 0.01
Crude fiber	10.27 $\pm$ 0.03
Acidity (as acid malic %)	4.20 $\pm$ 0.20
pH	2.55 $\pm$ 0.01
Bx ^o	12.51 $\pm$ 0.02

All values are means of triplicate determinations $\pm$ standard deviation (SD)

2.1. Analysis of phenols & antioxidant activity:

Total phenolic content (TPC) of the Roselle calyces obtained using different solvent systems is presented in Table 2. Results obtained in the present study revealed that the level of TPC in the extracts of roselle calyces was considerable (Table 2). The mean TPC of the roselle calyces was shown to decrease in the order of ethanol: methanol > ethanol > methanol > water extracts. This indicated that phenolic compounds of roselle plants are better extracted with ethanol: methanol than the other solvents.

Table 2: Extraction yield, TPC, DPPH & β -carotene analyses of roselle calyces extracts.

	Water	Methanol :water(50-50)	Ethanol:water(50-50)	Methanol:ethanol(50-50)
Extraction yield (%)	35.5 ^C	33.82 ^D	40.48 ^B	46.41 ^A
TPC(mg GAE/g)	24.36 ^D	36.94 ^C	39.38 ^B	44.43 ^A
DPPH (%)	43.85 ^D	80.27 ^C	86.67 ^B	91.77 ^A
β -carotene (%)	39.45 ^D	53.20 ^C	57.87 ^B	60.43 ^A

^{A, B, C, D} In each line, different letters means significant differences ($p < 0.05$)

The difference is probably due to the characteristic of the solvent; this could affect which compounds are extracted from the plant matrix. This phenomenon can be explained by a change in polarity of the antioxidant

compound due to the particular solvent used for extraction. The amounts of TPC in the roselle calyces extracts (as shown in Table 2) are similar to that found by Mohd-Esa *et al.*, (2012).

Abou-Arab *et al.*, (2011) obtained that dried roselle calyces contained 37.42mg/g dry weight sample of total phenolic compounds. According to Salazar-Gonzales *et al.*, (2012), the presence of polyphenol compounds could account for the reasonably strong antioxidant activity in the roselle extracts.

The solubility of phenolic compounds is governed by the chemical nature of the plant, as well as the polarity of the used solvents. The polar character of anthocyanins makes them soluble in several types of polar solvents such as methanol, ethanol, acetone and water. The current study agrees with results found by Christian and Jackson (2009) and Anokwuru *et al.*, (2011). Even though ethanol is less efficient and more difficult to eliminate it would be preferred for food, because methanol is toxic. The results are in agreement with those reported by Shil *et al.*, (2005), that the ethanol combinations are good solvents for polyphenol extraction and are safe for human.

The antioxidant activity of a sample is strongly dependent on the model system in which it is evaluated. A single analytical assay (β -carotene bleaching method) may be inadequate for measuring antioxidant activity. For this reason, the extracts of roselle calyces were also examined for their radical-scavenging capacities using the stable radical, DPPH, which has been widely used to test the free radical-scavenging ability of various chemicals. As shown in Table 2, DPPH radical-scavenging activities of the extracts decreased in the order: ethanol: methanol > ethanol > methanol > water.

Antioxidant activity of the Roselle extract correlated strongly to its anthocyanin content (Tsai *et al.*, 2002). According to Wong *et al.*, (2002), the roselle extract is an electron donor and can react with free radicals to convert them into more stable products and terminate radical chain reactions. Antioxidants are significant in the prevention of human illness and may function as free radical scavengers, (Andlauer and Furst, 1998).

2.2 HPLC analysis:

Fig.1 shows the chromatogram roselle calyces extracts. The HPLC chromatogram reveals that delphinidin - 3-O-sambubioside, cyanidin-3-O-sambobioside are the major phenolic compounds in roselle calyces extracts. These are may be mainly responsible for the antioxidant activity. In previous studies, it is known that, anthocyanin is the major source of antioxidant capacity in roselle calyces extract and reported chromatograms similar to that depicted in Figure. Since roselle rich in anthocyanin , so that it could be another potential source of antioxidant and as well as good natural food colourant especially for producing a brilliant red colourant for many food .

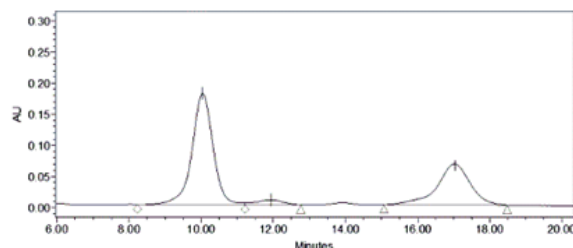


Fig.1: Fig.1. HPLC chromatogram of Roselle calyces extract (a): delphinidin -3-O-sambubioside, (b): cyanidin-3-O-sambobioside.

2. Conclusion:

An extraction agent for roselle calyces also plays a major role in an antioxidant activity of the extract. The present study indicated that both aqueous and ethanol extracts from the calyx *H. sabdariffa* L. have significant natural phenols content and antioxidant activity. The results of the current study clearly demonstrated that alcoholic extracts of *H. sabdariffa* L. contain highest phenolic compounds than aqueous extract. This result indicates that ethanol: methanol (50-50) is better solvent than other solvents for extraction of phenol from *H. sabdariffa* L. calyces and this result agrees with Anokwuru *et al.*, (2011).

Roselle extract is rich in anthocyanins, and could be used as a good source for producing a brilliant red colourant for many foods (Clydesdale *et al.*, 1979 and Pouget *et al.*, 1990). Since roselle rich in anthocyanin , so that it could be another potential source of antioxidant and as well as good natural food colorant especially for producing a brilliant red colorant for many food .

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