Physico- chemical properties of natural pigments (anthocyanin) extracted from Roselle calyces (*Hibiscus subdariffa*)

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Abstract: Physico-chemical properties of Roselle calyces (*Hibiscus subdariffa*) indicated that moisture content, protein, fat, fiber and ash were 12.81 %, 7.51%, 0.46 %, 11.17 % and 11.24 %, respectively. Mineral contents of K, P, Na Ca, Mg, Fe, Zn, Cu and Mn were detected at different levels. The results showed that the Roselle calyces powder more the red color. Besides, contained ascorbic acid (140.13 mg/100g), total anthocyanins (622.91 mg/100g) and total phenolics (37.42 mg/g dry weight). The DPPH scavenging capacity obtained from raw dried Roselle was 36.53 µ/ml. The extraction of natural pigments (anthocyanins) from Roselle calyces by different solvents (ethanol acidified with 1.5N/L HCl (85:15, v/v), ethanol acidified with 1% citric acid, citric acid. solution of 2% concentration and distilled water) were applied and pigments were analyzed for color, pH, total acidity, total soluble solids (T.S.S), total anthocyanins, total phenolic and antioxidants activity. The obtained results indicated that the highest yield of pigment recovered is considered the main goal in the extraction process. In addition to economic considerations, safety should be considered. Accordingly, water acidified with citric acid 2 % indicating anthocyanins yield of 1063 mg/100 g might be the best choice and the more preferable solvent compared with ethanol acidified with HCl which showed the highest yield i.e. 1386 mg/100 g dry weight. The results from this study showed that the greater the Roselle extracted by 2 % citric acid solution the more the red color intensity observed (a* 5.25). Results of these studies can be used to determine application of Roselle anthocyanins in a variety of food products as food colourants such as confectionery products, gelatin desserts, snacks, cake, pudding, ice cream and beverages.

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1. Introduction:

Food colourants are either natural or synthetic depending on source. Natural colourants are extracted from renewable sources such as plant materials, insects, algae, etc, while the synthetic colourants are manufactured chemically and are the most commonly used dyes in the food, pharmaceutical and cosmetic industries. Due to this limitation and worldwide tendency towards the consumption of natural products, the interest in natural colourants has increased significantly (**Huck and Wilkes, 1996**). Of special interest to the food industry is the limited availability of red pigments (**Lauro and Francis, 2000**), therefore research into natural sources of red pigments have increased recently.

Due to perceived safety and physiological advantage of the natural colourants over synthetic ones, interest are being geared into search of new natural colourants and the verification of the safety of existing ones. *Hibiscus sabdariffia* (also known as Roselle) is a tropical plant of considerable economic potential. Its calyces have been suggested as food colourants for food industries; emulsifier for carbonated drinks, jam manufacture, juices and natural food colourants (**Duangmal *et al*., 2004**). The calyces are rich in anthocyanin, ascorbic acid and hibiscus acid. It is water soluble with brilliant and attractive red colour and with sour and agreeable acidic taste which aid digestion. The other health benefits of this plant include diuretic and choloratic properties, intestinal antiseptic and mild laxative actions. It also used in treating heart and nerve disorder, high blood pressure and calcified arteries (**Asolkar *et al*., 1992**).

Color is one of the most important quality attributes affecting the consumer's acceptance of food since it gives the first impression of food quality. Many convenience foods such as confectionery products, gelatin desserts, snacks, cake, pudding, ice cream and beverages would be colorless, and would thus appear undesirable without the inclusion of colorants (**Hirunpanish *et al*., 2006**). In this respect, Roselle calyces appear to be good and promising sources of water soluble red colorants that could be utilized as natural food colorants.

Roselle (*Hibiscus subdariffa* L.) is a tropical plant which belongs to the family *Malvaceae* and is

known in Egypt as *Karkadah*. It is probably a native of West Africa and is now widely cultivated throughout the tropics and subtropics e. g. Sudan, China, Thailand, Egypt, Mexico, and the West India (**El-Saidy *et al*., 1992**).

In addition, Roselle juice, which is conventionally made from water extraction of fresh or dried Roselle calyxes, has been reported as being a popular soft drink with daily consumption in many countries including Egypt, Sudan, Mexico, Nigeria and Thailand (**Aurelio *et al*., 2007**).

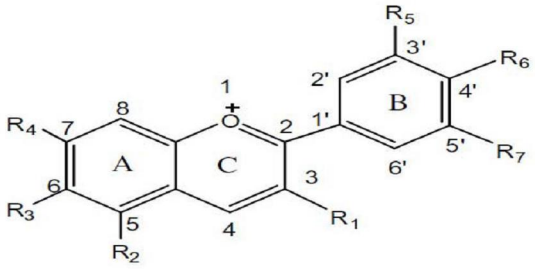
The chemical components contained in the flowers of *Hibiscus sabdariffa* include anthocyanins, flavonoids and polyphenols **(Tzu-Li Lin *et al*., 2007).** The petals are potentially a good source of antioxidant agents as anthocyanins and ascorbic acid **(Prenesti *et al*., 2007).** Roselle calyx contains a rich source of dietary fiber, vitamins, minerals and bioactive compounds such as organic acids, phytosterols, and polyphenols, some of them with antioxidant properties. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, sambubioside, and cyanidin-3- sambubioside mainly contributing to their antioxidant properties (**Aurelio *et al*., 2007**). Recently, the biological activities of anthocyanins, such as antioxidant activity and anticarcinogenic activity have been investigated (**Tsai *et al*., 2002**).

The flowers of *Hibiscus sabdariffa* are rich in anthocyanins (**Cisse *et al*., 2009**). The anthocyanins (**Figure 1**) are responsible for the red color, while the acid taste is due to the presence of some organic acids. Sepals' acidity may also contribute to their color variation.

Colour stability of anthocyanins depends on a combination of various factors including: structure of anthocyanins, pH, temperature, oxygen, light and water activity. Enzymatic degradation and interactions with food components such as ascorbic acid, sugars, metal ions, sulfur dioxide and copigments are no less important (**Jackman and Simith, 1996**).

It is used effectively in folk medicines for treatment of hypertension, inflammatory diseases and cancer **(Tzu-Li Lin *et al*., 2007)**. The calyces are used to decrease blood viscosity and reduce hypertension **(Christian *et al*., 2006).** Hibiscus pigments reduce the incidence of liver lesions including inflammation, leucocyte infiltration and necrosis **(Kong *et al*., 2003).**

The antioxidant activity of Roselle extract is pH dependent (pH 2 to 7), the activity decreases as pH increases. However, at a constant pH, only a relatively small decrease in antioxidant activity and total phenolic content is observed **(Sukhapat *et al.*, 2004).**



R1 = O-Sugare (glucose, arabinose, galactose)
R2, R4, R6 = OH
R3 = H and R5, R7 = H, OH, OCH3

Figure 1. Structure of anthocyanins

The objectives of the present study were: extraction of natural pigments (anthocyanins) from natural plant sources such as Roselle calyces (*Hibiscus subdariffa*) by different solvents and determination of the efficiency of the solvents in the extraction of Roselle pigments. The main purpose of this study was to focus on determining the physico-chemical properties of the major anthocyanins present in Roselle calyx. Results of these studies can be used to determine application of Roselle anthocyanins in a variety of food products as food colourants.

2. Materials and Methods

Materials:

Raw materials

-Calyces of Roselle (*Hibiscus subdariffa* L.) were used as source of the natural pigments investigated in the present study. The dried calyces of Roselle were purchased from a local market in Cairo, Egypt.

-The dried Roselle calyces were ground for 3 second using a blender (Braun KMM 30 mill), type 3045, CombiMax (Germany). The dried calyces were immediately packed in polyethylene bags and kept at low temperature (4°C) till used.

Chemicals

All chemicals used were at least analytical grade. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and citric acid were obtained from Sigma Chemicals Co. (St. Louis, USA). Anhydrous sodium carbonate (Na2CO3), Folin–Ciocalteu phenol reagent, hydrochloric acid (HCl), methanol, ethanol, ascorbic acid, sodium hydroxide and indophenols were obtained from Merck (Darmstadt, Germany).

Mineral standards

Standard solution (1000 ppm) of macro elements; potassium (K),calcium (Ca), sodium (Na), and magnesium (Mg) as well as micro-elements; copper (Cu), manganese (Mn), iron (Fe) and zinc (Zn) were provided by Merck (Darmstadt, Germany). The standards were prepared from the individual

1000 mg/L (Merck).Working standards were prepared from the previous stock solutions.

Extraction of anthocyanins:

Four solvents were compared in the extraction of pigments from Roselle calyces. The solvents evaluated were:

1. Ethanol acidified with 1.5N/L HCl (85:15, v/v).
2. Ethanol acidified with 1% citric acid.
3. Citric acid solution of 2% concentration.
4. Distilled water.

Extraction pigments were carried out according to the procedures described by **Du and Francis (1973) and Pouget *et al*. (1990).** Roselle calyces were removed from the refrigerator and ground into powders in order to increase the surface area for pigment extraction.

A suitable quantity (4.0 g) of Roselle calyces powder were immersed in 100 ml of the tested solvent and kept and 4 °C overnight. The mixture was filtered through a filter paper (Whatman No. 1) and the residue on the filter paper was re-extracted for four times with suitable quantity of the solvent and water till the filtrate became almost colorless. The filtrates were collected and combined in a 500 ml volumetric flask and made up to volume with the extracting solvent.

Extraction of pigment:

Roselle pigments were extracted with solvent according to **Mattuk (1998).** One hundred gram of ground dried calyces were thoroughly mixed with a suitable amount of solvent in 500 ml flask and incubated overnight in the refrigerator at 4 °C. The soluble extract was filtered using Whatman No. 1 filter paper to obtain Roselle pigment extract. The properties of dried Roselle extract were determined:

Analytical methods:

Physical analysis:

Determination of color:

The color characteristics including **(i)** lightness (L), **(ii)** redness/greenness (± a), and (iii) blueness/yellowness (± b) of the calyces were measured by using a Hunter Lab. Model D25 color and color difference Meter according to **Hunter method (1958).** The machine was calibrated using black and white tiles and the samples were analyzed in triplicate.

Determination of pH:

The pH of Roselle calyx were measured using pH meter (model Cyber Scan 500) standardized with buffer solutions of 4.0 and 7.0 according to the method of **AOAC (2000).**

Total titratable acidity (TTA):

Total titratable acidity was expressed as % citric acid was determined by standard **AOAC (2000)** using 0.1 N NaOH and phenolphthalein as an indicator.

The total soluble solids (T.S.S):

The total soluble solids (T.S.S) (^{o}Brix) of samples was determined according to **A.O.A.C (2000)** at room temperature (25 ºC± 1) expressed as ºBrix (0 - 90), was determined with a land Refractometer (ATAGO, Japan).

Chemical analysis:

Moisture, protein, fat, ash and fiber were determined according to the methods described in the **AOAC (2000).** Total carbohydrates calculated by differences.

Mineral contents, that is, copper (Cu), magnesium (Mg), manganese (Mn), iron (Fe) and zinc (Zn) were determined on aliquots of the solutions of the ash were established according to the method of **AOAC (2000)** using Atomic Absorption Spectrophotometer, Perkin-Elmer Model 2380 manufacture (USA). The flame photometer was applied for calcium (Ca), potassium (K) and sodium (Na) determination according to the method described by **Pearson (1976).**

Determination of ascorbic acid:

Ascorbic acid (vitamin C) was determined according to the indophenols method according to the indophenols method (**AOAC, 2000**).

Determination of Anthocyanins:

Total anthocyanins content of Roselle extract was determined colorimetrically according to the procedure described by **Du and Francis (1973)** where a known volume of the filtered extract was diluted to 100 ml with the extracting solvent. The colour intensity was measured at wave length of 520 nm for water and citric acid solution extracts and 535 nm for acidified ethanol using Spectrophotometer (model T80 x UVNIS Spectrometer PG Instruments Ltd) . The total anthocyanins content referred to cyanidin-3-glucoside was calculated using the following equation:

Total anthocyanins (mg/100g) =

$$\frac{\text{Absorbance X dilution factor X 100}}{\text{Sample weight X 55.9}}$$

Determination of total phenol:

Total phenolic content (TPC) was determined using the Folin-Ciocalteu assay (**Kahkonen *et al*., 1999**). Samples (300 µl; triplicate) were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before absorbance at 765 nm was measured. TPC was expressed as gallic acid equivalent (GAE) in mg per 100g of dry material. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($r^2 = 0.9998$), where y is absorbance and x is concentration of gallic acid in mg/l.

Total antioxidant activities:

Antioxidant activities of extracts measured included radical-scavenging activity**.** The methods were based on procedures described by **Chan *et al.* (2007).** Radical-scavenging activity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Different dilutions of extracts (1 ml) were added to 2 ml of DPPH (5.9 mg/100 ml methanol). Absorbance was measured at 517 nm after 30 min. Radical-scavenging was calculated as EC_{50} and expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg ascorbic acid /100 g.

AEAC (mg AA/100 g) = IC_{50} (ascorbate)/ IC_{50} (sample) × 10^5

The EC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml

Statistical analysis:

The data obtained from study was statistically subjected to analysis of variance (ANOVA) and means separation was by **Snedecor and Cochran (1980).** The least significant difference (L.S.D) value was used to determine significant differences between means and to separate means at $p< 0.05$ using SPSS package version 15.0.

3. Results and Discussion

Chemical analysis of raw materials:

The results shown in Table (1) indicated that moisture content of naturally dried Roselle calyces was 12.81 %. Chemical analysis also showed that Roselle calyces contained 7.51, 0.46 and 11.17 % of protein, fat and fiber, respectively. Results given in the same table indicated that Roselle calyces had high ash content which reached 11.24 %. 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**).

Table1. Chemical analysis of Roselle calyces (*Hibiscus subdariffa*) (% dry weight basis)

Constituent	% dry weight basis
Moisture	12.81±0.02
Protein	7.51±0.02
Fat	0.46±0.03
Crude fiber	11.17±0.02
Ash	11.24±0.02
Total carbohydrates*	69.62±2.0

*Total carbohydrates: calculated by difference

-All values are means of triplicate determinations ± standard deviation (SD).

The results are in agreement with those reported by (**Adenipeku, 1998**) who showed that the calyces contain 11.33 % moisture and 6.90 % protein. The results indicate the nutritional content of calyces compared well literature value. Typical literature values are; carbohydrates (68.75 %), protein (6.71 %) and fat 1.01 %). This may be attributable to the source of calyces (**Ameh *et al*., 2009**).

Mineral composition of raw materials:

Table 2 shows the mineral composition of the Roselle calyces. In the minerals considered in this study K, Ca, and Mg had the highest value, Fe and P had the medium value, Na, Zn, Cu and Mn had the least value, while Ni and Co were not detected.

Table 2. Mineral composition of raw Roselle calyces (% dry weight basis)

Element	Macro-elements (mg/100g) ±SD
Potassium (K)	20.60±0.02
Phosphorus (P)	36.22±1.0
Sodium (Na)	6.62±0.02
Calcium (Ca)	912.15±2.0
Magnesium (Mg)	315.21±1.0
	Micro-elements (mg /100g)
Copper (Cu)	4.32±0.02
Manganese (Mn)	2.39±0.03
Zinc (Zn)	6.51±0.03
Iron (Fe)	37.80±1.0
Nickel (Ni)	ND
Cobalt (Co)	ND

-All values are means of triplicate determinations ± standard deviation (SD).

ND**:** Not detection

The trend indicates that the consumption of the Roselle calyces will took and active role in good bone and teeth formation and could be useful in blood formation.

Apart from giving the desirable color to the food, the colourants added reasonable quantity of K (2060 mg/100g) and Na (6.62 mg/100g) that is required to maintain osmotic balance of the body fluid and the pH of the body, muscle regulation and nerve irritability, glucose absorption control and enhancement of normal retention of protein during growth (**Food and Nutrition Board, 2000**). Similar quantities of these minerals had earlier been reported in vegetable minerals (**Aremu *et al*., 2005, 2006**).

Though the concentration of Fe and Zn were 37.80 and 6.51 mg/100g, the quantities are available for biochemical function. The daily recommended Fe requirements for humans are 10-15 mg for children, 18 mg for women and 12 mg for men (**Oluwaniyi *et al*., 2009**).

Zinc plays an important role in human nutrition. Zinc deficiency results in retarded growth and delayed sexual maturation because of its role in nucleic acid metabolism and protein synthesis **(Underwood, 1971).** Therefore, sufficient intake of minerals and trace elements such as iron, zinc and calcium are important for ensuring optimal health, growth, and development of infants and young children

(Untoro *et al*., 2005)

The cupper was (4.32 mg/100g), the daily requirement is only 2 mg. Fe and Cu are present in cytochrome oxidase (enzyme) which is involved in energy metabolism (**NAS, 1976**). Calcium is a coordinator among inorganic elements, for example excess amount of K, Mg or Na in the body can be corrected by Ca and also adequate quantity of Ca in the diet assist in Fe utilization (**Fleck, 1976**). The Mg and Ca value in the Roselle calyces are high which has 315.21 and 912.15 mg/100g, respectively. Mg is an activator of many enzymes systems maintains the electrical potential in nerves (**Shills and Young, 1992**).The mineral content of plants can be significantly influenced by variety, location, and environmental conditions **(Rao, 1996**).

Chemical and antioxidant properties of dried Roselle:

Chemical and antioxidant properties of Roselle calyces powder are shown in Table 3. From the results, CIE LAB color was measured with the following color coordinate lightness (L*), redness (a*, red-green) and yellowness (b*, yellow-blue). L*, a* and b* values. L*, a* and b* values of Roselle were 28.3, 71.0 and 45.9, respectively. The results from this study showed that the Roselle calyces powder the more the red color a* (71.0).

It was found that the dried Roselle contained the ascorbic acid, total anthocyanins as cyanidin 3-glucoside and total phenolics content which recorded 140.13 mg/100g, 622.91 mg/100g and 37.42 mg/g dry weight sample, respectively. The pH value was 2.73, % total acidity as citric acid was 18.85 and 13.5 °Brix total soluble solids (T.S.S). The DPPH scavenging capacity obtained from raw dried Roselle was 36.53 µ/ml (Table 3).

Table 3. Chemical and antioxidant properties of dried Roselle calyces (on dry weight basis)

Properties of dried Roselle	**(on dry weight basis) ±SD**
Color	
L*	28.3±1.0
a*	71.0±2.0
b*	45.9±2.0
pH	2.73±0.02
Titratable acidity (% as citric acid)	18.85±2.0
Total soluble solids (°Brix)	13.5 ±1.0
Ascorbic acid (mg/100g)	140.13±3.0
Total anthocyanin content (as cyaniding-3-glucoside (mg/100g)	622.91±2.0
Total phenolic content as gallic acid (mg/g)	37.42±2.0
DPPH, IC_{50} (µ/ml)	36.53±2.0

-All values are means of triplicate determinations ± standard deviation (SD).

It is well known that Roselle extract is characterized by its sour taste. This is confirmed by the total acidity of Roselle calyces which was as high as 18.85 %. In the same ways, pH value was a low as 2.73.

The results in (Table 3) are consistent with the fact that calyces are rich in vitamin C (141 mg/100g). This may be attributable to the source of calyces (**Wong *et al*., 2002**). This indicated that Roselle has a higher content of ascorbic acid than

guava, orange and mango. (**Tee *et al.*, 1997)**. Vitamin C content of Roselle calyces is related to the state of freshness or dryness.

Wills *et al.* (1998) reported that the pH value of Arab Roselle was 2.62. The pH depends on the concentration of free H ions or mirrored the changes in total organic acids. The free state of H ions is due to dissociation of H ions from the carboxylic group (- COOH) of organic acid. This increase in pH throughout maturation was due to a metabolic process in the fruits that resulted in the decrease of organic acids. This is because organic acids are an important source of respiratory energy in plant cell.

The amount of phenolic compounds in the raw Roselle was 37.42 mg/g dry weight (Table 3) is similar to that found by **Mazza, *et al.* (1999)** in various fruits such as strawberries and currants. Ascorbic acid and other phenolic compounds are good for health maintenance and prevention of disease (**Ames *et al.*, 1993**).

Extraction efficiency of anthocyanins from Roselle calyces as affecting by the type of solvents:

Several solvents were compared in order to use the most effective one for extracting the pigments for Roselle calyces.

Ethanol acidified with 1.5 N/L HCl (85:15), ethanol acidified with 1% citric acid, 2 % citric acid solution and distilled water were used in extracting the pigments from Roselle calyces. The yields of pigments recovered with the different solvents are shown in Table (4). Addition of acids to water or ethanol increased the efficiency of anthocyanins extraction compared with distilled water alone. In general HCl was more effective than citric acid. Ethanol acidified with HCl, showed the strongest influence on the amount of anthocyanins extracted, followed by 2 % citric acid solution and ethanol acidified with 1 % citric acid.

The same results showed that the yields of anthocyanins, using ethanol acidified with HCl, ethanol acidified with1 % citric acid and 2 % citric acid solution, were 1386, 1063, and 693 mg anthocyanins per 100 g Roselle calyces, respectively. While distilled water extracted only 545.39 mg/100 g dry weight. Similar data were reported by **Mattuk (1998).**

It could be concluded that adding acid to the extraction medium had a great effect in stabilizing anthocyanins, thus the extraction efficiency increased. The highest yield or content of natural anthocyanins pigment was obtained when extraction was performed using acidified ethanol while the extraction by water only gave the minimum yield. Acidification with HCl serves to maintain a low pH, thereby providing a favorable medium for the formation of flavylim chloride salts from simple anthocyanins and improving the efficiency of anthocyanins extractions. However, the use of mineral acids such as HCl may alter the native form of complex pigments by breaking associations with metals or copigments **(Mattuk 1998).**

Table 4. Extraction efficiency of anthocyanins from Roselle calyces as affected by the type of solvent

Extracting solvents	Yield of anthocyanins* (mg/100 g)
Ethanol acidified with 1.5 N/L HCl (85: 15)	1386
Ethanol acidified with 1 % citric acid	693
2 % citric acid solution	1063
Distilled water	545.39

*Based on dry weight

These observations reveal that the pH value is a very important factor affecting the extraction of anthocyanins indicating that at lower pH values anthocyanins yield was the highest. **Bronnum-Hansen and Flink (1985)** reported similar results where they noted that the efficiency of extracting solvent increased with increasing the concentration of citric acid and concluded that pH of extracting medium was the determining factor for anthocyanins extractability.

Extraction of anthocyanins is commonly carried out under cold conditions with methanol or ethanol containing a small amount of acid with the objective of obtaining the flavylium cation form, which is red and stable in a highly acid medium. However, acid may cause partial hydrolysis of the acyl moieties in acylated anthocyanins, especially in anthocyanins acylated with dicarboxylic acids such as malonic acid **(Bronnum- Hansen and Flink 1985).**

Ethanol is another good solvent for polyphenol extraction and is safe for human consumption (**Shi1 *et al.*, 2005**). In preparing anthocyanin-rich phenolic extracts from plant materials, an acidified organic solvent, most commonly methanol or ethanol is used. This solvent system denatures the cell membranes, simultaneously dissolves the anthocyanins, and stabilizes them. However, care should be taken to avoid addition of excess acid which can hydrolyze labile, acyl, and sugar residues during concentration steps. To obtain

the best yield of anthocyanin extraction, weak organic acids, such as formic acid, acetic acid, citric acid, tartaric acid and phosphoric acid, and low concentrations of strong acids, such as 0.5-3.0% of trifluoroacetic acid and < 1.0% of hydrochloric acid are recommended (**Nicoue *et al*., 2007**). In addition, sulfured water has also been used as extraction solvent in seeking a reduction of the use of organic solvents as well as the cost of extraction **(Cacace and Mazza, 2002).**

The polar character of anthocyanins makes them soluble in several types of polar solvents such as methanol, ethanol, acetone, and water. Solvent extraction of anthocyanins is the initial step in the determination of total and individual anthocyanins prior to quantification, purification, separation, and characterization **(Rivas-Gonzalo, 2003)** and generally involves the use of acidified methanol or ethanol. Even though ethanol is less efficient and more difficult to eliminate later, it would be preferred for food use, because methanol is toxic. The use of acid stabilizes anthocyanins in the flavylium cation form, which is red at low pH **(Rivas-Gonzalo, 2003).** However, solvent acidified with hydrochloric acid may hydrolyze acylated anthocyanins, which explains why it has been overlooked in the past that many anthocyanins are acylated with aliphatic acids **(Strack, and Wray, 1989).** To avoid or at least minimize the breakdown of acylated anthocyanins, organic acids such as acetic, citric, or tartaric acids, which are easier to eliminate during anthocyanin concentration, have been preferred (**Strack, and Wray, 1989**)

The extraction of anthocyanins using ethanol acidified with citric acid (0.01%) instead of hydrochloric acid was reported by Ethanol would be preferred for food use to avoid the toxicity of methanolic solutions. Citric acid is less corrosive than hydrochloric acid, chelates metals, maintains a low pH, and may have a protective effect during processing (**Timberlake and Bridle, 1980**).

The extracting solution should be slightly acidic to maintain the flavylium cation form, which is red and stable in highly acidic medium, but not too acidic to cause partial hydrolysis of the acyl moieties in acylated anthocyanins **(Andersen and Markham, 2006).**

The main objective of the present experiment was to close the solvent that is more suitable in pigment extraction from Roselle calyces on commercial applications. In fact, the highest yield of pigment recovered is considered the main goal in the extraction process. However, in addition to economic considerations, safety should be considered. Accordingly, water acidified with citric acid 2 % indicating anthocyanins yield of 1063 mg/100 g might be the best choice and the more preferable solvent compared with ethanol acidified with HCl which showed the highest yield i.e. 1386 mg/100 g dry weight.

Physico- chemical properties of Roselle plant extracted by different solvents:

Color:

Table 5 shows physical and chemical properties of Roselle plant extracted by different solvents. From the results, CIE LAB color was measured with the following color coordinate lightness (L*), redness (a*, red-green) and yellowness (b*, yellow-blue). L*, a* and b* values of Roselle extracted by ethanol acidified with 1.5 N HCl (85:15) were 2.72, 4.79, 1.81, respectively; L*, a* and b* values of Roselle extracted by ethanol acidified with 1 % citric acid were 2.00, 3.36 and 1.33, respectively; L*, a* and b* values of Roselle extracted by 2 % citric acid solution were 3.43, 5.25 and 1.74, respectively and L*, a* and b* values of Roselle extracted by distilled water were 2.08, 3.23 and 1.39, respectively. The results from this study showed that the greater the Roselle extracted by 2 % citric acid solution the more the red color intensity observed (a* 5.25) followed by ethanol acidified with 1.5 N HCl were (a* 4.79). It was found that the distilled water extraction to less brilliant red in color (a* 3.23) and also less the amount of total anthocyanin contents.

It could be concluded that the Roselle extracted by 2 % citric acid solution showed that the more the red color intensity. The results showed that the Roselle calyces contained natural constituents of organic acid such as malic, citric and 3-indlyl acetic acids (**AL- Khtani and Hassan, 1990**) which played an important role in giving brilliant red color of sample extract

pH value and titratable acidity (as citric acid %):

The results in Table (5) showed that the pH was in the range of 2.70 to 2.82.

It is well known that Roselle extract is characterized by its sour taste. The extraction by 2 % citric acid solution exhibited high acidity were 19.82 %, followed by ethanol acidified with 1 % citric acid (18.93 %). This increase in acidity may be due to the extraction of anthocyanin by citric acid Table (5).

Total soluble solids (TSS):

As seen in Table (5), the Roselle plant extracted by different solvents exhibited varying degree of total soluble solids. The ethanol acidified with 1.5 N HCl (58:15) extracts from the Roselle plant exhibited higher total soluble solids (T.S.S) than did the other solvents. Total soluble solids content were (20 °Brix), followed by ethanol acidified with 1 % citric acid (16 °Brix) and 2 %

citric acid solution were (12 °Brix) .While the distilled water extraction to less in T.S.S (5 °Brix).

Anthocyanins:

The anthocyanin pigments content recovered with the different solvents are shown in Table (5). In general HCl, was more effective than citric acid. Ethanol acidified with 1.5 N HCl, showed the strongest influence on the amount of anthocyanins extracted (1386 mg/100g dried Roselle), followed by 2 % citric acid solution (1063 mg/100g dried Roselle) and ethanol acidified with 1% citric acid (693 mg/100g dried Roselle). While distilled water extracted only 545.39 mg/100g dried Roselle).

The results are in agreement with those reported by **Mattuk (1998)** reported that the highest yield or content of natural anthocyanin pigment was obtained when extraction was performed using acidified ethanol followed by acidified water then ethanol while the extraction by water only gave the minimum yield.

It has been demonstrated that in acidic media, four anthocyanthin structures, including the flavylium cation, the quinonoidol base, the carbinol pseudbase and chalcone, exist in quilibrium. And at pHs below 2, the anthocyanthin exists primarily in the form of the red flavylium cation. As the pH is raised (≥ 4.5), a rapid portion loss occurred to yield blue quinonoidal forms (**Mazza and Miniati, 1993**).

Total phenols:

As plant polyphenolic constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, the total amount of phenolic compounds in the Roselle extracts was determined using the Folin- Ciocalteu method. to obtain a crude estimate of the amount of phenolic compounds present in an extract. phenolic compounds respond differently to the Folin-Ciocalteu reagent, depending on the number of phenolic goups they have (**Singlton *et al*., 1999**). As shown in Table (5), all extracts contain high amount of content, to which their antioxidant activity may be ascribed. As seen in Table (5), the Roselle plant extracted by different solvents exhibited varying degrees of total phenolic content. The ethanol acidified with 1% citric acid extracts from the Roselle plant exhibited higher total phenolic than did the other solvents.

Total phenolic content were (45.06 mg/g as gallic acid), followed by ethanol acidified with 1.5 N HCl, (85:15) were (42.00 mg/g as gallic acid) than did the other solvents the difference is probably due to the characteristics of the solvent: this could affect which compounds are extracted from the plant mature. As the polarity of the solvent increases, higher extraction yields of total extractable phenolic compounds were obtained were obtained **(Esa *et al*., 2010).**

The results are in agreement with those reported by **(Shil *et al*., 2005)** reported that the ethanol is good solvent for polyphenol extraction and is safe for human. In preparing anthocyanin rich phenolic extracts from plant materials, an acidified organic solvent, most commonly ethanol or methanol is used.

Previous studies have shown that the developmental stage of the plant may affect biosynthetic pathways of phenolic compounds this could then affect the total contents (**Krizman *et al*., 2007**).

Antioxidants:

DPPH free radical scavenging activity

The stable DPPH radical has been used widely for the determination of primary antioxidant activity that is the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 517 nm. In this study, the antioxidant activity was expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg ascorbic acid /100 g. of plant material on a dry basis.

As seen in Table (5), the Roselle plant extracted by different solvents exhibited varying degrees of antioxidant activity. The ethanol acidified with 1% citric acid extracts from the Roselle plant exhibited higher value in total antioxidant activity expressed as the lowest of the amount of sample (µ/ml) needed for 50 % decrease of the initial DPPH concentration (EC_{50}) were 42.77 (µ/ml), which was significantly greater antioxidative effect than those other solvents followed by ethanol acidified with 1.5 N HCl, (85:15) were (43.18 µ/ml). The difference is probably due to the characteristics of the solvent: this could affect which compounds are extracted from the plant mature. This phenomenon can be explained by a change in polarity of the antioxidant compounds due to particular solvent used for extraction. As the polarity of the solvent increases, higher extraction yields antioxidant compounds **(Esa *et al*., 2010). Christian and Jackson (2009)** reported that the high antioxidant activity observed in the Roselle could be due to the high ascorbic acid content of this Roselle.

Preferably formed under acidic conditions, these weak chemical associations can augment anthocyanin stability and increase antioxidant properties (**Molien-Aubert *et al*., 2001**).The antioxidant activity of Roselle extract is also pH dependent (pH 2 to 7), the activity decreased as pH increases. However, at a constant pH, only a relatively small decrease in antioxidant activity content is observed (**Sukhapat *et al*., 2004**). **Tee et**

al. (2002) assessed the antioxidant properties of Hibiscus by comparing its activity with those of BHA and β-carotene. The results showed that the Hibiscus extract had stronger antioxidant activity than BHA or tocopherol in a linoleic acid model system.

Duh and Yen (1997) reported that the calyces of Roselle possessed high contents of phenolic compounds. The water extract of calyces also showed good hydrogen donating abilities, indicating that they had effective activities as radical scavengers. It is known that Roselle calyces samples are very rich in vitamin C, anthocyanins, polphenols and other water-soluble antioxidants (**Duke and Atchley, 1984**).The antioxidant properties of herbs may be attributed to the plant pigments that are the main components of each herbal extract. The red pigment presented in the flowers of Hibiscus species are anthocyanins (cyanidin-3-glucoside and delphinidin-3- glucoside) which may act as an antioxidant (**Ho, 1992**).

Hibiscus sabdariffa L. which contained high phenolic compounds exhibited high antioxidant activity when determined by DPPH assays. It, thus, confirms that phenolic compounds have an important role in antioxidant activities (**Harborne, 1998**). A good correspond between antioxidant activity and phenolic compounds were found in Bulgarian medicinal plants (**Ivanova *et al*., 2005**).

4. Conclusion

The obtained results indicated that the highest yield of pigment recovered is considered the main goal in the extraction process. In addition to economic considerations, safety should be considered. Accordingly, water acidified with citric acid 2 % indicating anthocyanins yield of 1063 mg/100 g might be the best choice and the more preferable solvent compared with ethanol acidified with HCl which showed the highest yield i.e. 1386 mg/100 g dry weight. The results from this study showed that the greater the Roselle extracted by 2 % citric acid solution the more the red color intensity observed (a* 5.25). Results of these studies can be used to determine application of Roselle anthocyanins in a variety of food products as food colourants such as confectionery products, gelatin desserts, snacks, cake, pudding, ice cream and beverages.

Table 5. Physico- chemical properties of Roselle plant extracted by different solvents (±SD)

Properties of dried Roselle extract	Ethanol acidified with 1.5 N HCl (85:15)	Ethanol acidified with 1 % citric acid	2 % citric acid solution	Distilled water	LSD at 5 %
Color					
L*	2.79±0.03^{b}	2.00±0.02^{c}	3.43±0.03^{a}	2.08±0.02^{c}	0.02
a*	4.79±0.02^{b}	3.36±0.03^{c}	5.25±0.02^{a}	3.23±0.03^{d}	0.02
b*	1.81±0.02^{a}	1.33±0.03^{d}	1.74±0.03^{b}	1.39±0.03^{c}	0.02
pH	2.82±0.03^{a}	2.70±0.03^{c}	2.71±0.02^{bc}	2.73±0.02^{b}	0.02
Titratable acidity (as citric acid %)	11.58±0.02^{b}	18.93±1.01^{a}	19.02±1.0^{a}	18.85±1.0^{a}	1.64
Total soluble solids (°Brix)	20.00±2.0^{a}	16.00±1.0^{b}	12.00±1.0^{c}	5.00±0.03^{d}	1.88
Total anthocyanin content (as cyanin-3- glucoside mg/100g dried Roselle calyces)	1386±4.0^{a}	693±3.0^{c}	1063±3.0^{b}	545.39±2.0^{d}	17.88
Total phenolic contents, as gallic acid (mg/g)	42.00±2.0^{a}	43.06±1.0^{a}	41.72±2.0^{a}	38.39±2.0^{b}	2.49
DPPH, EC_{50} (µ/ml)	43.18±2.0^{b}	42.77±2.0^{b}	44.53±2.0^{ab}	45.64±2.0^{a}	1.88

-All values are means of triplicate determinations ± standard deviation (SD).
- Means within rows with different letters are significantly different ($P < 0.05$).
EC_{50}**:** The amount of sample (µ/ml) needed for 50 % decrease of the initial DPPH, concentration

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