Physico Chemical Properties and Antioxidant Activity of Roselle Seed Extracts

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Abstract: The aim of this research was to extract phenolic compounds from defatted Roselle (Hibiscus sabdariffa L.) seed and assess their antioxidant potential. Water, ethanol (30%), methanol (30%) and acetone (30%) were used as solvent for extraction. The proximate composition, total phenolic content and extraction yield were analyzed. Antioxidant efficacies of Roselle seed extract were tested by using 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH), hydroxyl, 2, 2’-azinobis-3-ethylbenzothiaz oline-6-sulfonic acid (ABTS) radicals scavenging capacities and reducing power analysis. Roselle seeds were found to be rich in protein (27.745%), carbohydrates (40.45%) and oil (30.83%). The total phenolic content ranged from 1.66±0.03 to 1.99±0.01 (GAE) mg/g using water and 30% acetone reducing power analysis. Roselle seeds were found to be rich in protein (27.745%), carbohydrates (40.45%) and oil (30.83%). The total phenolic content ranged from 1.66±0.03 to 1.99±0.01 (GAE) mg/g using water and 30% acetone reducing power analysis.

INTRODUCTION

Hibiscus sabdariffa L. or Roselle is one of the most common flower plants grown worldwide. The origin of H. sabdariffa L. is not fully known, but it is believed to be native to tropical Africa. Roselle is used to produce jam, jelly and wine. Omobuwajo et al. (2000) reported that Roselle seeds are bigger than pearl millet varieties, having average dimension of 5.21 and 2.81 mm. The seeds are somewhat bitter. However, in Africa, they are ground into meal for human food due to their high protein content. They are also roasted to use as a substitute for coffee (Morton, 1987) and contain a substantial amount of oil that resembles that of cotton seed (Mohammed et al., 2007).

In ethno-medicine, H. sabdariffa L. is used to deal with several health problems, including hypertension, pyrexia, liver disorders and microorganism growth. It is also used as a diuretic, sedative, or digestive (Faraji and Tarkhani, 1999; Chen et al., 2003; Akindahunsi and Olaleye, 2003) and is said to have aphrodisiac properties (Duke and DuCellier, 1993). Many medicinal applications of this plant have been developed around the world. Studies by Muhammad and Shakib (1995) have shown that Roselle can prevent cancer, lower blood pressure and improve the digestive system in humans. Its calyx extract has also been used as an effective treatment for patients with kidney stones due to its uricosuric effect (Prasongwatana et al., 2008).

Antioxidants are bioactive micro-components present in food, having the ability to retard oxidation. Free radicals have been implicated for aging as well as food and chemical deterioration. Many researchers found that plant polyphenolic compounds scavenge free radicals, thereby protect humans against oxidative damage and diseases (Beecher, 1999; Cicerale et al., 2009; Okawa et al., 2001). Recently, natural antioxidant α-tocopherol and some synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and propyl gallate are commonly used to act against free radicals in food and biological systems. However, synthetic antioxidants are under heavy criticism due to their undesirable side effects on human health (Goli et al., 2005). In order to minimize or eliminate the use of these artificial antioxidants in food, essential oils or plant extracts have been used as natural antioxidants against the proliferation of microorganisms or for protecting food from oxidation (Bubonja-Sonje et al., 2011). For example, Roselle plant protects against Low Density Lipoprotein (LDL)-oxidation and has hypolipidemic effects in vivo (Hirunpanich et al., 2006). In some instances, it is also used to preserve food. There are many reports published on the efficacy of Roselle.
Preparation of defatted roselle seed flour: Roselle seeds were cleaned by removing dust, stones and plant debris. The seeds were milled using a laboratory scale hammer mill and the resulting powder was sieved through a 60 mesh screen until fine powder was obtained. Thereafter, the powder was defatted with n-hexane, following a small-scale hexane extraction method described by Tzeng et al. (1990). The oil-free flour was desolventized and stored in a desiccator at room temperature for subsequent use.

Proximate analysis: Proximate analysis is an important index for identification and classification of the nutritional value of a food material. The seeds are regarded as a by-product of Roselle leaves processing and used in West Africa as raw material for making Bikalga known as datou in Mali, a kind of fermented condiment.

The proximate analysis of Roselle seed was determined according to AOAC (2000). The moisture content was determined by drying in an oven at 105°C until a constant weight was obtained. Ash was determined by weighing the incinerated residue obtained at 550°C for 8-12 h. Total nitrogen and protein content were determined based on the Kjeldahl method using the conversion factor of 6.25. The total lipid in samples was determined by Soxhlet method. The total available carbohydrate was determined by difference, subtracting the sum of percentage of moisture, crude fat, crude protein and ash contents from 100%. Triplicate samples were analyzed for each sample.

Minerals composition: Samples were digested in 100 mL micro-Kjeldahl flask with HNO₃/HClO₄ until the solution became colorless. The samples were cooled and diluted to volume in a 25 mL volumetric flask with 0.1 M HCl. Sodium, potassium, calcium, magnesium, iron, zinc, manganese and copper were measured by atomic absorption spectrophotometry, (Garcia et al., 1972) using a Varian spectra atomic absorption spectrophotometer (Varian SpectrAA220, Varian, Palo Alto, CA).

Fatty acid analysis: Fatty acids content in Roselle seeds were determined according to the method of Ceirwyn (1995). Fat was extracted with methyl ether and prepared directly by treatment with sodium methoxide. Gas Chromatography/Mass Spectra (GC/MS) system was used to identify and quantify the fatty acids. A FINNIGAN TRACE MS gas chromatograph/mass spectra equipped with a 30 m×0.25 mm Ov-1701 column was used. Column flow rate was 0.8 mL/min with helium as the carrier gas, split was 64 mL/min and the source temperature was 270°C. The fatty acid methyl esters were identified by comparison with the retention times of NU CHECK Inc. standards (Elysian, 1L) and quantified by internal normalization.

Polyphenol extraction: Polyphenolic extract yields of plant matter are substantially influenced by the nature of the extracting solvent owing to the presence of different bioactive compounds of different chemical properties and polarities that may or may not be soluble in a particular solvent. Thus differences in polarity coupled with safety reasons influenced the choice of the four solvents used for the study.

The extraction was carried out in a 250 mL beaker by the following method: (to 20 mL of different solvents: de-ionized water, 30% acetone, 30% ethanol and 30% methanol (v/v) was added 1 g of defatted Roselle seed flour). Laboratory scale magnetic stirrer was used to gently stir. The phenolic extracts were separated from the solid mass by filtration using Whatman’s No. 1 filter paper. Each filtrate obtained was collected into a graduated cylinder and the volume adjusted to 20 mL with the same extracting solvent in order to estimate the total polyphenol content and assay for antioxidant activity.

Total Polyphenolic compound content determination (TP): The amount of total polyphenol content in the Roselle seed extracts was calorimetrically estimated using the Folin-Ciocalteu reagent method according to Sultana et al. (2009), with slight modification. Briefly, 0.04 mL of each crude extract was mixed with 0.2 mL Folin-Ciocalteu reagent in a 10 mL test tube. The mixture was allowed to stand for 5 min before addition of 0.5 mL sodium carbonate (20%). The volume was adjusted to 5 mL with distilled water and vortexed for 10 sec. The mixture was incubated at 45°C for 30 min and cooled to room temperature before reading the absorbance at 750 nm using a UV spectrophotometer (Model: UV-1800, China-Shanghai
The amount of TP contents was expressed as Gallic Acid Equivalent (GAE) mg/g Roselle seed on dry weight basis. All samples were analyzed three times and the results were averaged.

**Scavenging effect on hydroxyl (HO•) radical:** The hydroxyl radical scavenging effects of Roselle seed extract was assayed using the method of Halliwell et al. (1987) with slight modification. The reagents were added to a test tube in the following order: 0.4 mL KH2PO4-KOH buffer (pH 7.4), 0.1 mL sample solution with concentrations (10, 15, 20 mg/mL) and 0.1 mL of 1 mM EDTA, 10 mM H2O2, 60 mM 2-deoxy-Dribose, 2 mM ascorbic acid and 1 mM FeCl3 (0.1 mL distilled water was used as control instead of FeCl3). The reaction solution was incubated at 37°C for 1 h. The reaction was stopped by addition of 1 mL of 20% TCA. The color was developed by addition of 1 mL of 1% TBA into the reaction tubes, which were placed in boiling water for 15 min. The tubes were cooled to room temperature and then the absorbance was read at 532 nm. For each concentration, samples were prepared in triplicate and the antioxidant activity of each was measured in duplicate. The scavenging effects were calculated by the following equation:

Radical scavenging activity (%) = (1 - Ac/As) ×100

where,
As: The absorbance of the samples at 560 nm
Ac: The absorbance of the control at 560 nm

**Reducing power:** The reducing power of Roselle seed powder extract was measured according to the method of Wu et al. (2003) with some modifications. Briefly, freeze dried samples at concentrations of 0.5, 1 and 1.5 mg/mL were dissolved in a 0.2 M phosphate buffer (pH 6.6). An aliquot (2.5 mL) of sample solution was then added to 2.5 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50°C for 20 min, followed by addition of 2.5 mL of 10% (w/v) Trichloroacetic Acid (TCA). After incubation 2.5 mL of distilled water and 2.5 mL of 0.1% (w/v) FeCl3 were added to the mixture. The absorbance of the solution was then recorded immediately at 700 nm. The blank of each sample was prepared by adding distilled water instead of FeCl3. Net increase in absorbance of the reaction mixture indicates increased reducing power.

**DPPH radicals scavenging activity assay:** The scavenging effect of Roselle seed powder extract on DPPH free radical was measured according to the method of Shahidi et al. (2006) with little modification.

Freeze-dried samples were dissolved in 0.1 mM DPPH, initially dissolved in 95% ethanol at serial concentrations of (1, 2 and 3 mg/mL). An aliquot (0.1 mL) of each sample solution was added to 1.9 mL of ethanolic DPPH solution (50 µM) and the mixture was shaken and left for 30 min at room temperature. The absorbance of resulting solution was read at 517 nm. A lower absorbance represents a higher DPPH scavenging activity. The scavenging effect was expressed as shown in the following equation:

DPPH scavenging activity (%) = 100%× [Abscontrol - (Abssample - Absblank) /Abscontrol]

where, Absample is the absorbance of the different Roselle seed extracts with DPPH, Abscontrol is the absorbance of DPPH without any Roselle seed extracts, Absblank is absorbance of Roselle seed extracts without DPPH. The EC50 value (the median effective concentration that causes a decrease in the initial radical concentration by 50%) is a parameter widely used to measure the antiradical efficiency. The lower the EC50 value, the higher the free radical-scavenging ability (Zhang et al., 2006).

**ABTS radicals scavenging activity assay:** ABTS radical scavenging activity was determined as described by Phanturat et al. (2010). ABTS free radical (ABTS•+) was generated by reacting 7.4 mM ABTS and 2.6 mM potassium persulphate at a ratio of 1:1 (v/v). The mixture was stored in the dark for 12 h at room temperature. Prior to assay, ABTS•+ solution was diluted with methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm. To initiate the reaction, 150 µL of freeze dried sample at different concentrations (2, 4 and 6 mg/mL, respectively) was mixed with 2850 µL of ABTS•+ solution. The absorbance was then read at 734 nm after 2 h of incubation in dark, at room temperature. The blank was prepared in the same manner, except that distilled water was used instead of the sample. The percentage reduction of ABTS•+ to ABTS was calculated according to the following equation:

ABTS radical scavenging activity (%) = [(Acontrol - A sample) /Acontrol] ×100

**Statistical analysis:** All analyses were performed in triplicate. Data were analyzed using Statistical Package for Social Science (SPSS) version 17.0 software package. Results were analyzed statistically using a 2 sample test, assuming equal variances and one-way Analysis of Variance (ANOVA). The difference was considered significant if p<0.05.

**RESULTS AND DISCUSSION**

**Proximate chemical composition:** The proximate analyses of dried whole Roselle seed extracts are presented in Table 1. From the results, solid content was the most important factor. The carbohydrate, fat, protein, ash and moisture content were 40.45, 20.83, 27.32, 4.67 and 6.79%, respectively.
Table 1: Proximate composition of roselle seeds powders

<table>
<thead>
<tr>
<th>Parameter (%)</th>
<th>Mean±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>27.32±0.38</td>
</tr>
<tr>
<td>Moisture</td>
<td>6.79±0.14</td>
</tr>
<tr>
<td>Fat</td>
<td>20.83±0.55</td>
</tr>
<tr>
<td>Ash</td>
<td>4.67±0.21</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>40.45±0.60</td>
</tr>
</tbody>
</table>

Mean values in rows are significantly different (p<0.05); S.D.: Standard deviation

Table 2: Minerals composition of roselle seeds powders (μg/g)

<table>
<thead>
<tr>
<th>Parameter (μg/g)</th>
<th>Mean±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc</td>
<td>120.74±0.21</td>
</tr>
<tr>
<td>Potassium</td>
<td>19799.33±0.58</td>
</tr>
<tr>
<td>Iron</td>
<td>80.17±0.49</td>
</tr>
<tr>
<td>Magnesium</td>
<td>4113.11±0.76</td>
</tr>
<tr>
<td>Sodium</td>
<td>434.32±2.83</td>
</tr>
<tr>
<td>Calcium</td>
<td>2155.08±0.55</td>
</tr>
</tbody>
</table>

Mean values in rows are significantly different (p<0.05); S.D.: Standard deviation

Table 3: Fatty acids analysis of roselle seeds oils (contents are area %)

<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>Roselle seed oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
</tr>
<tr>
<td>Myristic acid</td>
<td>0.2411</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>18.7637</td>
</tr>
<tr>
<td>Arachidic acid</td>
<td>0.5910</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4.8624</td>
</tr>
<tr>
<td>Total</td>
<td>24.4500</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>0.3233</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>33.0752</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>35.1653</td>
</tr>
<tr>
<td>α-linolenic acid</td>
<td>1.4539</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
</tr>
</tbody>
</table>

Fatty acid analysis: Fatty acid composition of Roselle seed oil is depicted in Table 3. The seeds contained a considerable amount of fatty acids. The total content constituted of 70% unsaturated and 24.45% saturated fatty acids. Oleic and linoleic acids had the highest values of 33.07 and 35.16%, respectively. Among the unsaturated fatty acids (Table 3), palmitoleic acid content was the lowest (0.32%), whereas palmitic acid (18.76%) was the highest saturated fatty acids (Table 3). The fatty acid composition shown the high content in unsaturated fatty acid, especially linoleic acid (35.16%), thus, is indicating the nutritional benefits of Roselle seeds. Linoleic acid has beneficial effects on blood lipids, lowering blood pressure and serum cholesterol (Savage, 2001; Enujiugha and Akanbi, 2008). The test showed no significant difference (p<0.05) among the sample in terms of fatty acid content and composition.

Total phenolic content: Results obtained in this study reveal that the level of total phenolic compounds in Roselle extracts is considerable (Fig. 1).

Total phenolic content of different Roselle extract varied significantly (p<0.05) ranging from 1.99±0.01 to 1.66±0.03 mg GAE/g for 30% acetone and water extract respectively. The extract prepared using 30% acetone showed the highest phenolic content, while the lowest was observed in the aqueous extract with 1.99±0.01 and 1.66±0.03 mg GAE/g, respectively with a significant difference (p<0.05) when compared with other tested solvent extracts (Fig. 1). This study showed that Roselle seed extracts obtained using different solvents, had different total phenolic contents. The differences observed in yields could be related to the polarity of particular solvent used in the extraction. Acetone (30%) yielded the highest contents and water the lowest. From these results, the yields of phenolics followed the following order: 30% acetone>30% ethanol >30% methanol >water. The results are in agreement with the findings of John et al. (2012) for cocoa shell polyphenols. This indicates that phenolic compounds of Roselle seed are better extracted with acetone than all the other solvents tested.

Hydroxyl radical scavenging activity: Hydroxyl radical is an extremely reactive oxygen species, capable of modifying almost every molecule in the living cells. In addition, hydroxyl radicals are capable the quick initiation of lipid peroxidation process by subtracting hydrogen atoms from unsaturated fatty acids (Kappus et al., 1991; Aruoma, 1998). The ability of Roselle seed extracts to scavenge hydroxyl radicals was evaluated and results are shown in Fig. 2. All extracts were capable of scavenging hydroxyl radicals. At a
concentration of 20 mg/mL, the 30% ethanol exhibited the strongest hydroxyl scavenging activity, followed by 30% methanol, 30% acetone and water extract. The hydroxyl radical scavenging activity was increased significantly (p<0.05) between 30% ethanol and 30% methanol extracts prepared from defatted Roselle seed powder. Roselle seed extracts obtained from ethanol, methanol, acetone and water extracts exhibited 66.36, 57.27, 56.36 and 30% hydroxyl radical scavenging activities respectively. These results show that Roselle seed extracts obtained from different solvents may exhibit an effective hydroxyl radical scavenging activity. Ethanol extract showed the higher OH scavenging activity (Fig. 2), but its ability to scavenge DPPH (Fig. 3) and ABTS (Fig. 4) was weaker than of the other solvent extracts. It could be that the phenolic compounds in different extracts had different mechanism against diverse free radicals scavenging activity. These results also demonstrated that 30% ethanol might be a more suitable solvent for extracting antioxidants from defatted Roselle seed powder resulting in higher hydroxyl scavenging activity, but weaker DPPH and ABTS scavenging abilities.

Reducing power: In this analysis, the presence of antioxidants caused the reduction of the Fe$^{3+}$/ferricyanide complex to the ferrous form and the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of each compound. Figure 5 shows the reducing power of different solvent extracts at different concentrations (0.5, 1, 1.5 mg/mL) from the defatted Roselle seed flour. Different solvent extracts showed significant (p<0.05) differences in their reducing power, indicating that Roselle seed reducing power can also be influenced by choice of extraction solvent. The reducing power of different solvent extracts increased with increase in amount of sample concentration (0.5, 1, 1.5 mg/mL). All the concentrations showed high activity. The reducing power of the different extracts was in the following order: 30% acetone >30% ethanol >30% methanol and water. This data indicates that Roselle seed extracts are capable of donating electrons, which can react with free radicals to convert them to stable products and strongly inhibit radical chain reaction.
values were 97.01, 91.16, 90.43 and 85.06% , acne >30% methanol >30% ethanol >water and the DPPH results, the highest and the lowest ABTS respectively at a concentration of 6 mg/mL. Similar to effect on ABTS radical was in the following order: 30% methanol, ethanol and water extract. The scavenging to the scavenging capacity of each sample: acetone, a cetone, a significant (p<0.05) in the concentration of ABTS due by four different solvent have ABTS radical scavenging other samples was significant (p<0.05). Similar results difference between the 30% acetone extract and the concentrations. Statistical analysis revealed that the extract had the highest scavenging activity at all were also found with respect to IC50 values as indicated in Table 4. Acetone had the least value (0.65 mg/mL), followed by methanol (0.69 mg/mL), water (1.24 mg/mL), and ethanol (0.84 mg/mL). A lower IC50 value means better efficiency of antioxidant activity of the sample. From these results acetone extract was the most potent solvent.

**ABTS radical scavenging activity:** ABTS scavenging activities of Roselle seed extracts are presented in Fig. 4. The results indicated that Roselle seed extracted by four different solvent have ABTS radical scavenging activity at the concentrations of 2-6 mg/mL. There was a significant (p<0.05) in the concentration of ABTS due to the scavenging capacity of each sample: acetone, methanol, ethanol and water extract. The scavenging effect on ABTS radical was in the following order: 30% acetone >30% methanol >30% ethanol >water and the values were 97.01, 91.16, 90.43 and 85.06%, respectively at a concentration of 6 mg/mL. Similar to DPPH results, the highest and the lowest ABTS scavenging activity was in the acetone and water extracts. Similar results were also found with respect to IC50 values (Table 4). Acetone had the least value (0.76 mg/mL), followed by methanol (1.68 mg/mL), ethanol (2.17 mg/mL) and water (2.49 mg/mL). It may be concluded that the acetone extract might be the appropriate solvent for extracting antioxidants with higher ABTS scavenging activity.

**CONCLUSION**

The results of this study show that Roselle seed is of high nutritional value and therefore can be a cheap potential source of many nutrients, especially protein and polyphenols. It was also revealed that 30% acetone effectively extracts polyphenols better than the other three solvents. Further investigations on the relationship between phenolic content and antioxidant activity of Roselle seed extract is needed, with particular emphasis on the identification of the bioactive compounds responsible for the biological activity.

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