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# IMPROVED EXTRACTION OF ANTHOCYANINS WITH ANTIBACTERIAL ACTIVITY FROM FRESH ROSELLE (*HIBISCUS SABDARIFFA* L.) CALYCES

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**Abstract**

Roselle (*Hibiscus sabdariffa* L.) flower is a commonly consumed vegetable in Bangladesh and have culinary value to preparation of soup, jelly, and baking. Since previous studies suggested the presence of anthocyanins in roselle calyces, this study was designed to enrich anthocyanins from the calyces of this flower. This study explored that addition of mineral acid during the extraction process further enriched the anthocyanins content of the fresh roselle calyces extract. Also, methanol acidified with 1% (v/v) HCl was found as the best solvent to extract anthocyanins from the calyces resulting 7.621±0.001 mg crude anthocyanins per gram of fresh sample. Anthocyanin extraction decreased if 1% (v/v) HCl was replaced with 10% (w/v) tartaric acid or 10% (v/v) acetic acid. The extracted anthocyanins showed minimal antibacterial activity. A further study will explore the possibility of use these anthocyanins for industrial purposes.

## Introduction

Flavonoids are one of the very large and widespread groups of plant secondary metabolites with potential bioactivities[(1)]. Anthocyanins are a type of flavonoids those differ from other flavonoids by strongly absorbing visible light[(2)]. Thereby, these compounds are intensely coloured. Also, anthocyanins are water-soluble pigments and responsible for all the red, pink, magenta, violet, and blue colours in the calyces, leaves, and fruits of higher plants[(3)]. Anthocyanins are enriched in fruits like cherries, berries, currants, nectarines and plums, as well as in almost all colourful edible flowers[(4,5)]. Plant anthocyanins are valuable compounds for two major reasons: their bioactivities and their industrial applications. Anthocyanins exert multiple effects on plants including stress tolerance, growth regulation, senescence and reproduction (pollination)[(6)]. And, these compounds show antioxidant and free radical scavenging activities, and anti-inflammatory, analgesic, antibacterial, and antihyperglycemic effects[(7)]. Such activities are helpful for the protection of anthocyanin-synthesizing plants and to development herbal medicines. Finally, anthocyanins have

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commercial value as they are used as an ingredient in many cosmetics, organic solar cells, beverages, colorants and nutraceuticals products[8]. The market share of anthocyanins is currently around 626 million USD and it is projected that the use of anthocyanins in industry will grow up to 5% eventually[3,5]. Anthocyanins are mostly biosynthesized either in corresponding plants or in metabolically engineered microorganisms, though few can be synthesized chemically. Till now most of the anthocyanins are commercialy produced by extracting from calyces or fruits of a native plant[8,9].

Roselle (*Hibiscus sabdariffa* L.) is a tropical plant which belongs to the family *Malvaceae* and commonly known as 'Meshta or Churuk' in Bangladesh[10]. The leaf of this plant is consumed as fried leafy vegetables and the calyces of the flowers are consumed as pickle or an additive to lentil soup in this country. The red intense color of the calyces of roselle flower is due to the presence of anthocyanins at around 2.5% of the dry weight[11]. A number of anthocyanins have been isolated from them. These anthocyanins include but not limited to hibiscin, chrysanthenin, gossypicyanin, and anthocyanidin[12]. These anthocyanins possess excellent biological and pharmacological activities including antioxidant, antimicrobial, antipyretic, hepatoprotective and nephroprotective activities[13]. Beside these, the anthocyanins of roselle have been applied in food technology as food grade coloring agent[14,15]. Color and structural stability of roselle anthocyanins depends on factors including the core structure of anthocyanins, pH, light, oxygen bioavailability, temperature, and water activity[16]. However, these anthocyanins are resistant to enzymatic degradation and do not interact with food components such as sugars, metal ions, co-pigments, ascorbates or sulphur derivatives[14,17].

Roselle anthocyanins have application in the field of beverages, dry gelatine mixes, and bakery products[11]. Although roselle grows well and in large quantities in Bangladesh mostly for its fibre (Mesta jute), the calyces of roselle have very limited use in food industries. Thereby, these calyces can be use as a source of food grade, anthocyanins-based coloring agents for different food industries of Bangladesh and as an ingredient for food, nutraceuticals and cosmetic industries. Limited marketing of dried calyces or aqueous extract of roselle calyces also exist in Bangladesh, but industrial production of roselle anthocyanins or extract is not in practice[3,10]. Commercial chemical synthesis of roselle anthocyanin is not viable and currently all roselle anthocyanins are isolated from their calyces by different extraction methods[9,18]. And, such process still requires optimization to prevent chemical modification, denaturalization and to preserve the intent color of the anthocyanin. The aim of this study is to further optimize extraction of food grade roselle anthocyanins for different industrial, household, and food biotechnology applications and to revalidate the antibacterial activity of such extracts. We found that acidified methanol is the best solvent to extract anthocyanins from roselle calyces with minimum antibacterial activity, though diluted tartaric acid solution can maximize the extraction of such anthocyanins for food industries.

## Materials and methods

*Sample Collection:* Roselle flowers were collected from local markets during the month of May. About 10 kg of samples were purchased. The calyces were separated from the flower immediately after purchase. Then the calyces were stored in -20 °C.

*Initial optimization of anthocyanins extraction:* Preliminary extraction of anthocyanins from fresh roselle calyces were done using method described previously with modification[19]. Briefly, 100 grams of calyces were smashed in 100 ml water and the suspension was kept 24 h in darkness at 4°C with occasional shaking. Then the solution was filtered and concentrated HCl was added to recolorize the solution at a final concentration of 1% (v/v). Then 100 ml chloroform was added to the solution and was shaken for 1 hour at room temperate in a closed bottle. The solution was then kept again for an hour to settle down for aqueous phase separation. Then the aqueous layer was separated using a separatory funnel. Anthocyanins content was measured during each step of the separation process.

*Optimization of solvents for anthocyanins extraction:* 100 grams of fresh calyces were smashed in 100 ml of either i) water, ii) water acidified with 1% (v/v) HCl, iii) water alkalinised with 1% (v/v) NaOH, iv) methanol, v) acidified methanol with 1% (v/v) HCl, vi) methanol alkalinised with 1% (v/v) NaOH, vii) ethyl acetate, or v) cyclohexane separately. The solutions were kept 24 h in darkness at 4°C with occasional shaking. Then the solutions were stand alone for an hour. After that, the upper phase was collected and was mixed with equal amount of chloroform. The aqueous layer was separated using a separatory funnel. The separated aqueous layer was then centrifuged for 15 min at 5000 rpm to remove any debris or suspended solids. Then the supernatant was collected and stored in darkness at 4°C.

*Quantification of anthocyanins:* Anthocyanins were detected and quantified using the procedure as described before[10,20]. Briefly, the absorbance of the supernatants was determined ranging 300-700 nm using UV-Vis spectrophotometer. The concentrations of the anthocyanins as mg/g weight of the calyces using the following equation:

$$\text{Total Anthocyanins} = [OD_{530} - (0.25 \times OD_{657})] \times TV / [(d\_wt) \times 1000]$$

where, OD = optical density; TV = total volume of the extract; d_wt = weight of the calyces in gram.

*Antibacterial activity assay:* Antibacterial assay was done by agar-well diffusion method using different gram negative bacteria[21]. *Escherichia coli*, *Shigella dysenteriae*, *Klebsiella pneumonia*, *Shigella boydii*, *Salmonella* Typhi, *Salmonella* Paratyphi and *Pseudomonas spp.*, and were inoculated to 5 ml of Nutrient Broth (NB) and incubated at 35-37°C for 4 to 5 hours until they rich $OD_{600}$ = 0.8. To find out antibacterial activity of the extracted anthocyanins, first the acidified anthocyanin extracts were neutralized to pH 7 by adding 1M NaOH, and then 10 µl was applied in each well of a Muller-Hinton agar plate spred with 100 µl bacterial cultures. Then the plates were incubated overnight at 37°C. After incubation, the antimicrobial activity of the test materials was determined by measuring the diameter of

the zones of inhibition in millimeter using a scale. Solvents neutralized to pH 7.0 was used as negative controls.

*Statistical analysis:* All statistical analysis was calculated using standard statistical methods in MS Excel™.

## Results and Discussion

Anthocyanins are valuable ingredients in food and beverages industries and enzyme, heat, and pH resistant anthocyanins are still in demand[8,17]. Hence, development of a method for improved extraction of anthocyanins from a common source was targeted in this study so that the anthocyanins yield can be maximized as much as possible. *Hibiscus sabdariffa* calyces are commonly consumed in Bangladesh and are considered as a good source of anthocyanins. Thereby, the calyces of *Hibiscus sabdariffa* were used as a source of natural anthocyanins. Previous studies found 1.2% (w/w) of anthocyanins using supercritical carbon dioxide as a solvent and 1.63% (w/w) anthocyanins using ethanol acidified with HCl from dried roselle calyces, though up to 2.5% (w/w) anthocyanins in dry roselle calyces was expected the best[19,22]. Even one study from Bangladesh showed maximum anthocyanin content of approximately 87.7 µg/g of dried calyces[10]. We hypothesized that a significant portion of anthocyanin could be lost during the drying process or during the extraction process from roselle calyces. To test this, we prepared anthocyanins enriched extract from fresh roselle calyces and track the anthocyanins content across the aqueous extraction process (Fig. 1). We found that addition of HCl to recolorize the initially obtained crude aqueous extract at 1% (v/v) final concentration, anthocyanin content in the extract prepared from fresh roselle calyces almost doubled. When chloroform was added to the recolorized extract, the anthocyanin content did not improve. When aqueous fraction and the chloroform fraction were separated from each other, almost all of the anthocyanins remained in the aqueous fraction and the chloroform fraction possibly contained other interfering pigments at a minimum level or there was a minimum loss of extracted anthocyanins in the chloroform fraction. Thus, we concluded that acidification of the initial crude extract is an important step towards the improvement of anthocyanins extraction from roselle calyces.

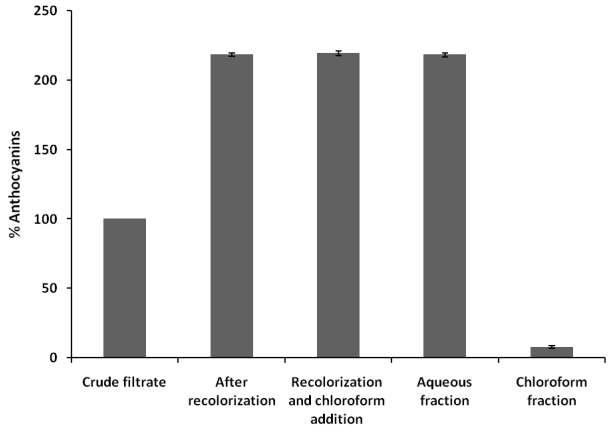


Fig. 1. Anthocyanin content of fresh roselle calyces across the extraction process. Data are represented as mean ± standard deviation (n = 3).

Next, we tested different solvent for further enrichment of anthocyanins in fresh roselle calyces extracts. For this, we tested water, water acidified with 1% (v/v) HCl, water alkalinised with 1% (v/v) NaOH, methanol, acidified methanol with 1% (v/v) HCl, methanol alkalinised with 1% (v/v) NaOH, ethyl acetate, and cyclohexane as solvent systems to compare the anthocyanins extraction efficiency. Table 1 summarizes the average yield of anthocyanins in these extracts of roselle calyces. It was observed that methanol acidified with 1% (v/v) HCl was the best solvent system to yield 7.621±0.001 mg crude anthocyanins per gram of raw fresh roselle calyces sample. Given that 89.4% of the fresh calyces are moisture, the anthocyanins yield from fresh roselle calyces by this way should be approximately 3.6% (w/w). This seemed to be around 1.44 times higher compared to the previous studies showing 2.5% (w/w) anthocyanins in roselle calyces[19,22]. This is possibly due to the fact that, a significant portion of anthocyanins could be lost during the drying process as most of the anthocyanins are heat sensitive and degrades over the time. Although methanol alone could extract almost similar amount of anthocyanins (7.28±0.002 mg per gram fresh source), the difference between anthocyanins yield with acidified methanol is still statistically significant ($p = 0.001$). However, cyclohexane failed to enrich any anthocyanins. Since anthocyanins are polar (water soluble) compounds, it is usual that cyclohexane could fail to extract such compounds from the source[9]. Also, enrichment of anthocyanins in extracts of fresh roselle calyces was poor in alkaline condition (1% (v/v) NaOH) as expected. This is because anthocyanins are not that much stable at high pH as they are at lower pH[23]. Efficiency of ethyl acetate to enrich anthocyanins from fresh roselle calyces was approximately half as compared to the aqueous extract. This finding suggested that ethyl acetate is not a good solvent to solubilise and stabilize anthocyanins extracted from roselle calyces.

**Table 1. Average content of anthocyanins in extracts of fresh *Hibiscus sabdariffa* L. calyces prepared with different solvent systems.**

| Solvent system | Anthocyanins (mg/g) | |
|---|---|---|
| | mean | SD |
| Water | 1.613 | 0.026 |
| 1% HCl in water | 3.631 | 0.015 |
| 1% NaOH in water | 0.843 | 0.003 |
| Methanol | 7.280 | 0.002 |
| 1% HCl in methanol | 7.621 | 0.001 |
| 1% NaOH in methanol | 1.111 | 0.006 |
| Ethyl acetate | 0.564 | 0.002 |
| Cyclohexane | 0.000 | 0.000 |

Thereby, the best extraction method to enrich anthocyanins from fresh roselle calyces was could be using methanol and 1% (v/v) HCl. However, since the HCl is not that much suitable for cosmetic and food industries, the extraction method was optimized with tartaric acid or acetic acid[17]. It was observed that changing the acid from HCl to 10% (w/v) tartaric acid or to 10% (v/v) acetic acid reduced anthocyanins content in the extract by 25% to 33% respectively, when methanol was used as the major solvent. But the anthocyanins content in fresh roselle calyces extracts reduced by 7% with water and 10% (w/v) tartaric acid and by 23% with water and 10% (v/v) acetic acid as compared to water and 1% (v/v) HCl (Fig. 2). However, extraction of roselle calyces anthocyanins as poor with water as compared to methanol. Thereby, it can be suggested that aqueous extraction with 10% (w/v) tartaric acid could be the best option for food and beverage purpose, despite low yield; and for other purposes methanol acidified with 1% (v/v) HCl could be the best solvent over all other solvent system tested here to extract roselle calyces anthocyanins.

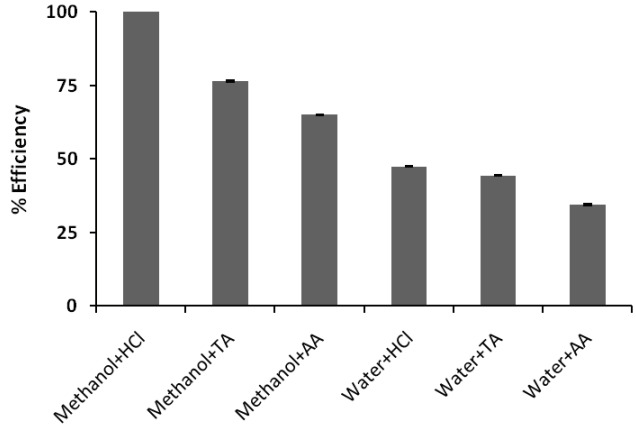


Fig. 2. Relative enrichment of anthocyanins from fresh roselle calyces extracts using organic acids as

acidifying agent. TA = Tartaric acid; AA = Acetic acid. Final concentration of the acid was 10% in all cases. Data represents comparison with methanol + 1% (v/v) HCl as a standard solvent.

It was previously shown that roselle anthocyanins possessed some degree of antibacterial activities[12]. Hence, to validate the potency of extract, antibacterial activity of the extract was justified. It was observed that the neutralized HCl-acidified methanolic and water-tartaric acid roselle calyces crude extract enriched with anthocyanins show minimal and comparable antibacterial activity on the tested bacteria (Table 2). We observed no growth inhibition for all the tested bacterial strain in negative controls, *i.e.* equal amount (10 µl) of either neutralized methanol-HCl solution or neutralized water-tartaric acid solution (not shown in Table 2). Both extracts failed to inhibit the growth of *Klebsiella pneumonia* and *Salmonella sp.* Typhi. And the best antibacterial activity was observed for *Shigella boydii* and *Shigella dysenteriae*. Hence, additional preservative might be required to increase the stability of the anthocyanins.

**Table 2. Antibacterial activity of roselle anthocyanins enriched extracts prepared with two different solvents.**

| Bacteria | Zone of Inhibition (mm) | |
|---|---|---|
| | **Methanol-HCl** | **Water-tartaric acid** |
| *Escherichia coli* | 6 ± 0.12 | 6 ± 0.5 |
| *Shigella dysenteriae* | 8 ± 0.18 | 9 ± 0.25 |
| *Klebsiella pneumonia* | ---- | ---- |
| *Shigella boydii* | 9 ± 0.50 | 9 ± 0.75 |
| *Salmonella sp.* Typhi | ---- | ---- |
| *Salmonella sp.* Paratyphi | 8 ± 0.14 | 8 ± 0.75 |
| *Pseudomonas spp.* | 7 ± 0.50 | 7 ± 0.50 |

Since our primary objective was to justify an industrial application of anthocyanins-enriched roselle calyces extract, we refrained ourselves from deducing the chemical composition or testing other biological activities of the extract. Also, cosmetics can be developed using this anthocyanin-rich fraction. For this, we'll try to stabilize anthocyanins in common cosmetics ingredients like beewax, castor oil and soybean oil. Further studies are required to improve such commercial products using the anthocyanins-rich roselle calyces extracts.

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