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Research

Antioxidant Compounds and Activities of Roselle (*Hibiscus sabdariffa* L.) Decoction Residues From Cordial and Juice Production

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ABSTRACT

Roselle is a popular plant in Malaysia, especially for its use in food and beverage production. The roselle calyces are decocted to make roselle juice and cordial, resulting in a significant amount of waste or roselle decoction residues. Surprisingly, these residues retain their dark red colour and are still intact although softened. However, to date, no research on the antioxidant compounds and activities of roselle decoction residues from cordial and juice production has been reported. Therefore, this study aims to determine the anthocyanins content and antioxidant activities remaining in the roselle decoction residues. The roselle decoction residues were evaluated for colour, Delta E (ΔE), pH, total anthocyanin content (TAC), total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging assay, ferric reducing ability assay (FRAP), and ferrous ion chelating (FIC). All assays were also correlated and showed positive effects among themselves. The decoction residues reported were still in acidic conditions ranging from pH 2 to 4. The decocted cordial residue (DCR) showed a similar colour with control or fresh roselle calyx (FRC), and a bit duller in decocted juice residue (DJR). The DCR showed similar and slightly lower antioxidant content and activity of TAC, TPC, TFC, DPPH, FRAP and FIC (73.34 ± 1.26 mg/100 g FW, 185.01 ± 15.27 mg GAE/g extract, 98.13 ± 5.79 mg QE/g extract, 19.88 ± 1.29 %, 209.22 ± 20.43 μM TE/g extract and 38.37 ± 1.18 %, respectively) to FRC but much better than DJR. The study revealed that the DCR residues were still rich in anthocyanin contents and had good antioxidant activity without having colour changes. These residues can be converted to wealth and could be an alternative source of natural antioxidants. Further research is needed to explore their potential applications in functional foods, dietary supplements, and pharmaceuticals.

Key words: Antioxidant activity, ethanolic extract, *Hibiscus sabdariffa*, roselle

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INTRODUCTION

In recent years, reducing food waste become a far-reaching problem and a rising issue globally. Around 14% of food produced is estimated lost in the post-harvest supply chain, but excluding the retail stage (FAO, 2019). According to FAO (2019), food waste includes food loss and the decrease in the quantity or quality of food. The food is lost or wasted throughout the supply chain, starting from the farm and progressing through processing, distribution, retail, and finally household consumption and/or dumping. As the actual data has not been documented yet, the earlier estimation of food loss and waste globally is around 1/3 every year (1.3 billion tons per year) (FAO, 2017). In terms of food groups, fruits, and vegetables (21.60%) reported a second higher level of loss after root, tubers, and oil-bearing crops (25.30%) followed by meat and animal products (11.90%), and cereals and pulses (8.60%).

Roselle (*Hibiscus sabdariffa* L.) belongs to the family

Malvaceae. It is reported native to Asia, from India to Malaysia or Tropical Africa and widely cultivated in almost all warm countries as a home garden crop including Malaysia (Musa *et al*., 2006). In Malaysia, roselle is a popular health drink among Malaysians, which is consumed for its high vitamin C and anthocyanin contents (Eslaminejad & Zakaria, 2011; Nur Amirah, 2015; Pacôme *et al*., 2019). These rich concentrations of vitamin C and anthocyanin contents have been documented in our previous report which was 229.53 mg/100 g fresh weight and 358.05 mg/100 g fresh weight, respectively (Nur Amirah, 2015). However, most studies emphasize the roselle calyx itself and its benefits to mankind, neglecting the wastes from roselle processing. Several types of roselle wastes are roselle calyx (from the beverage industry), leaves, seeds, and seedpod. It can be safely said that the waste from these popular health drinks mostly comes from cordial or juice productions. Roselle calyx that had been blanched from this process will be discarded as waste or called roselle decoction residue. According to a roselle beverage manufacturer in Terengganu, A. I. Agro Marketing, the roselle calyx is only blanched once in cordial and juice-making, then, the blanched roselle calyx will be discarded as waste or roselle decoction residue even though the deep red colour remains on the used calyx (Abdul Madik, personal communication, March 5, 2017). According to the manufacturer, the used calyces were not recommended to be recycled into producing the following batch of products because that will shatter the calyces into small particles producing cordial or juice with sediment (Abdul Madik, personal communication, March 5, 2017). In fact, to date, the waste generated from the blanched calyces created problems for the manufacturer and no effective waste mitigation has been employed. This industrial waste has become a major concern in the industry. Since plant wastes are generally prone to microbial spoilage, a lot of processes need to be considered before furthering the exploitation of wastes including drying, storage, and transportation. This exploitation of waste will increase the cost of production as well as possess an additional economic limitation to waste utilization. Hence, waste is not only a challenging problem for manufacturers or food processors, but they also become crucial at the international level due to both environmental pollution and economic aspects (Oreopoulou & Tzia, 2006; Baiano, 2014). Therefore, the manufacturers often used this industrial waste as feed and fertilizers (Oreopoulou & Tzia, 2006). The potentially valuable nutrients contained in the wastes are also expected to be lost. However, if the wastes from roselle processing can be reutilized into valuable products such as incorporation as food additives and supplements which may create an enhanced profit revenue, exploitation of the waste may then be an interest to the manufacturer.

Most of the research on roselle in Malaysia has so far been concentrated on the antioxidant content and activity, nutritional value, and health benefits of either fresh roselle or its products (Halimatul *et al*., 2007). However, the antioxidant content and activities of roselle by-products from both juice and cordial are scarce and not well documented. Hence, this study aims to determine the anthocyanins content and antioxidant activities remaining in the roselle decoction residues produced from cordial and juice processing. Mercado-Mercado *et al*., (2015) reported that the decoction residues or roselle wastes are a good source of dietary fibre and polyphenols with antioxidant capacity. The polyphenols bioaccessibility in decoction residue is 71.72%, and only 26.68% was recorded in roselle calyx. This indicates that the decoction process can increase polyphenols bioaccessibility in the gastrointestinal tract, in contrast with roselle calyx which remains higher proportion associated with soluble dietary fiber. The caffeic, gallic, and chlorogenic acids were found in polyphenols released from decoction residue (Mercado-Mercado *et al*., 2015). According to Sáyago-Ayerdi *et al*. (2007) and Sáyago-Ayerdi *et al*. (2009), only 66% of polyphenols detected in roselle samples were passed into roselle beverages after processing. They suggested that a relatively high amount of these precious compounds, approximately 34%, are left in the residue of this processing, which can be a source of wealth from residue.

Besides, previous studies have also reported the use of roselle calyx waste as a feed supplement for feeding livestock such as chicken broiler (Mohamed Aslam, 2018) and Japanese quail (Sinniah, 2018). According to EPA (2021), feeding animals is the third tier of EPA's Food Recovery Hierarchy. With proper and safe handling, farmers can use donated food scraps for livestock feeding, help the environment, and reduce costs (EPA, 2021). This has been supported by Rivin *et al*. (2012) and Jill Burkhardt (2020). They state that using food waste to feed the livestock can help farmers reduce feed costs and help food waste generators reduce the costs of disposal while minimizing the environmental impacts of this waste. The Food and Drug Administration (FDA) and the Department of Agriculture of the United States (USDA) are federal agencies that regulate the feeding of food waste to livestock (Rivin *et al*., 2012). The guidelines also have been developed for feeding alternative food waste and food by-products. This is due to not all livestock can take every food or residue from waste especially. For example, protein concentrate from potato fruit water can only be fed to cattle due to its high potassium content (Laufenberg *et al*., 1996), olive cake (Clemente *et al*., 1997) and sugarcane bagasse (Purchase,

1995) also not recommended for feeding due to its low digestibility (Laufenberg *et al*., 2003). Hence, proper selection of suitable livestock and the amount of concentration should be considered well for feeding livestock with waste derived from roselle.

In addition, a study using roselle seeds as food supplements to feed the quail birds was conducted. The study revealed that feeding quail birds with roselle seeds as a food supplement did not affect the meat's tenderness but resulted in better carcass characteristics (Md Yusof, 2018). Roselle seeds also have potential to become a source of dietary fiber as reported by Nyam *et al*. (2012). Cookies incorporated with roselle seeds powder shown improved antioxidant properties as well as can be used as a dietary fiber source, substituting fat without losing their quality. Halimatul *et al*. (2007) also mentioned that the roselle seeds grown in Malaysia have a good protein quality like casein. In addition, the roselle seed extracts used in the patties proved to possess some antioxidative bioactive compounds that can inhibit the lipid peroxidation in cooked beef patties (Mohd-Esa *et al*., 2010), justifying the prospective exploitation of roselle wastes. These reported findings are interesting and help to justify that roselle calyx waste has potential antioxidants and antimicrobial compounds that can be reutilized based on persistent colour retention. Therefore, the problem can be approached with a broader perspective by using extraction, purification, and valorization in different fields including food, cosmetics, pharmaceutical, and chemical industries. It is believed that through the extraction process, the available compounds from roselle calyx wastes can be recovered.

The exploitation of co-products (Baiano, 2014) can be done by using the waste valorization process, the process of reusing, recycling, or composting waste materials and converting them into more useful products (AIChE, 2020). The valorization can be attained through the extraction of high-value components such as proteins, polysaccharides, fibres, flavour compounds, and phytochemicals, which later can be reused as nutritionally and pharmacologically functional ingredients (Baiano, 2014). Hence, the roselle calyx waste needs to be extracted first before being further utilized. The biologically active compounds and phenolics derived from the waste extraction later can be used in various applications and industries (Lourith & Kanlayavattanakul, 2013). Therefore, the quantification of the active compounds needed to be done first to enable full utilization of roselle residues later.

MATERIALS AND METHODS

Plant material

Fresh roselle calyces variety Terengganu (UMKL-1) were purchased from Aslah Hibiscus, Aslah Supply and Services, Batu Pahat, Johor, Malaysia (4.2105°N, 101.9758°E) in January 2018. The study took place between January 2018 and March 2018. On the same day, the harvested roselle calyces were sent to the Postharvest Laboratory, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Terengganu (5.3117°N, 103.1324°E). The calyces were selected based on uniform shape, maturity, weight and free from damages and blemishes.

Experimental design

Cordial and juice are two main products in the roselle beverage industry. Therefore, in this study, the residue of the decocted calyces from cordial and juice making were collected and used as the treatments. Hence three treatments were involved; i) fresh roselle calyx (FRC) as the control, ii) decocted cordial residue (DCR), and iii) decocted juice residue (DJR) with five replications, each replication comprised of two experimental units. The study was laid in a complete randomized design (CRD). The FRC and the residues were used directly after being shredded into small pieces. The extraction used was a cold extraction maceration method. All analyses were carried out at laboratories, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Terengganu, and central laboratory, Universiti Malaysia Terengganu, Terengganu. The null hypothesis of this study is the roselle decoction residues do not contain significant levels of valuable compounds with antioxidant properties. This null hypothesis suggests that there is no difference in the presence of valuable compounds between the roselle decoction residues and FRC. The potential risk of a Type I error might occur when wrongly rejecting the null hypothesis and claiming there are significant differences, even when there are none. It could mean wrongly concluding that roselle decoction residues contain valuable compounds as compared to the FRC when, in reality, they do not. Given that the roselle decoction residues retain their deep red colour, it is plausible to contain valuable compounds. Hence, careful consideration and validation are essential to mitigate the risk of Type I errors. The p-value is used to examine type I errors the lower the *p*-value (0.05), the lower the likelihood of the type I error occurring.

Decocted sample preparation

The roselle calyces were decocted and then washed with water to remove the dirt and foreign matter. The roselle calyces were then cooked to produce cordial and juice imitating the real processing method provided by a manufacturer A.I. Agro Marketing Sdn. Bhd., Kuala Terengganu, Terengganu (5.3117°N, 103.1324°E), Malaysia. The cordial and juice were prepared by cooking the roselle calyces to water ratio of 1:1 and 1:2, respectively, until boiled. The residues of calyces used to produce the cordial and juice were noted as DCR and DJR. Later these residues were used to produce the ethanolic crude extract. Crude extract from FRC was also prepared as the control.

Crude extract preparation

To maximize the amount of extraction yields and retain their quality, the samples were extracted by using the maceration method and the residues were prepared prior, which are wet residues. The samples (FRC, DCR & DJR) were weighted (50 g) and put into a conical flask that had been fully wrapped with aluminium foil to minimize the exposure to light. The pure ethanol (95%) that was precooled at 5°C for 2 hr was added into the conical flask. Then, the conical flasks containing samples and pure ethanol (250 mL) were put in an orbital shaker (Stuart SSL1). The flasks were shaking constantly at 150 rpm for 24 hr at room temperature. Then, the macerated ethanol solutions were filtered with filter paper (Whatman no. 2, UK), and all extracted samples were collected in the wrapped laboratory glass bottle and stored at -20°C. The remaining samples that had been left in the conical flask, then, were extracted again twice and all the extracted samples were collected into the same wrapped laboratory glass bottle. After that, all the extracted samples were concentrated using a rotary evaporator (Buchi R-300EL) to obtain crude ethanolic extracts. The crude ethanolic extracts were then stored at -30°C prior to further analysis.

To counteract the potential repercussions of light-induced degradation, extraction procedures should be conducted in environments with minimal light or using methods that block light transmission. Therefore, during the extraction process, the conical flask was fully wrapped with aluminium foil to minimize exposure to light. It helps prevent light exposure and mitigate the risk of photochemical degradation during the extraction process. Moreover, optimizing extraction parameters like temperature, pH, and solvent choice can contribute to enhancing the stability of light-sensitive antioxidants in roselle extracts. Adopting these strategies can effectively preserve the antioxidant activity and quality of roselle extracts, ensuring their suitability for various applications across the food, beverage, and pharmaceutical sectors. Additionally, research conducted by Lourith & Kanlayavattanakul (2013) demonstrated that stable recovery of antioxidant colourants from roselle waste calyx, achieved through ethanol extraction at pH 3 and 23°C for 10 days storage, can be utilized for decorative purposes in pharmaceutical products such as cosmetics and as value-added components in food products.

Colour

Colour measurement in this study was conducted using a Konica Minolta Chroma-Meter via CIE L*, a* and b* systems. The samples were positioned on a Petri dish, and colour measurements were taken from the surface of the samples. The colour data were expressed in lightness (L*), a* value, b* value, hue angle (h°), and chroma (C*) (Nur Amirah, 2015).

Delta E (∆E)

The ∆E level was evaluated by the difference between the displayed colour and the original colour standard of the input content. It was measured on a scale from 0 to 100, where 0 is less colour difference, and 100 indicates complete distortion. Lower ∆E figures indicate greater accuracy, while high ∆E levels indicate a significant mismatch. The standard perception ranges (Schuessler, 2023) are < = 1.0 = not perceptible by the human eye, 1 - 2 = perceptible through close observation, 2 - 10 = perceptible at a glance, 11 - 49 = colours are more similar than the opposite, 100 = colours are exactly the opposite.

pH

The sample (2 g) was homogenized with 40 mL of distilled water using a juice extractor (handheld blender). The aliquots from extracted juice were determined by a glass-electrode pH meter using buffers of pH 4.0 and 7.0 for calibration (Nur Amirah, 2015).

Total anthocyanin content (TAC)

The TAC was determined using a modified method of Wan Zaliha (2009) and Nur Amirah (2015). The roselle calyx (0.2 g) was extracted by slicing the calyx into small pieces. Then, the sample was soaked in 10 mL of methanol (95%) and concentrated hydrochloric acid (HCl) in the ratio of (97:3 v/v)

within 18 hr at 2°C to 4°C in a wrapped beaker. After that, the extract was decanted and centrifuged at 5000 rpm for about 20 min at 4°C. The supernatant was determined at the wavelength 530 nm by using a UV-VIS Spectrophotometer (UV-1800, Shimadzu). The TAC was calculated for delphinidin-3-glucoside as described by Giusti & Wrosltad (2001). The data were expressed in mg/100 g of fresh weight. The calculation for anthocyanin pigment concentration as Equation 1. Where, A = Absorbance; MW = molecular weight, 465.2 g/moL for delphinidin-3-glucoside; DF = dilution factor; 10^3 = factor conversion from g to mg; ε = 34 700 cm^{-1}/mgL molar extinction coefficients for delphinidin-3-glucoside; 1 = pathlength in cm.

Equation 1:

$$\frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

Total phenolic content (TPC)

The TPC was determined by the Folin-Ciocalteau method, adapted from Swain & Hillis (1959) and Thaipong *et al*. (2006). The 150 µL of 1 mg/mL of crude ethanolic extract, 2400 µL of pure water, and 150 µL of 0.25 N Folin-Ciocalteau reagent were combined in a cuvette and mixed well using a vortex. The mixture was allowed to react for 3 min. Then, 300 µL of 1 N sodium carbonate, Na_2CO_3, solution was added to each cuvette and mixed well. The solution was incubated in the dark for 2 hr at room temperature (25°C). The absorbance was taken at 725 nm by UV-Vis spectrophotometer (UV-1800, Shimadzu) and expressed as gallic acid equivalents (GAE), mg GAE/g extract using gallic acid (0 mg/mL to 0.1 mg/mL) as standard.

Total flavonoid content (TFC)

The TFC was determined using the method described by Dewanto *et al*. (2002) and Abu Bakar *et al*. (2009) with slight modifications. A 500 µL of crude ethanolic extract (1 mg/mL) was combined with 2250 µL of distilled water, followed by the addition of 150 µL of 5% sodium nitrite, $NaNO_2$ solution into test tubes. After 6 min, 300 µL of 10% aluminium chloride, $AlCl_3 \cdot 6H_2O$, solution was added and allowed to react for 5 min before 1000 µL of 1 M sodium hydroxide, NaOH was added. The mixture was then mixed well by using a vortex. The absorbance was taken at 510 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu) and expressed as quercetin equivalents (QE), mg QE/g extract.

DPPH radical scavenging assay

The total antioxidant activity of roselle ethanolic extract against DPPH (2,2-diphenyl-2-picrylhyrazyl) radical scavenging assay was done according to the method of Brand-Williams *et al*. (1995) and Thaipong *et al*. (2006) with slight modifications. The DPPH stock solution was prepared freshly by dissolving 24 mg DPPH with 100 mL methanol (stored at -20°C until use). The DPPH working solution was prepared by mixing 10 mL stock solution with 45 mL methanol. 150 µL of each crude ethanolic extract (10 µg/mL) or methanol (roselle calyx) was dissolved in pure water and then allowed to react with 2850 µL of DPPH working solution. After vortexed, the samples and roselle calyx were then incubated at room temperature (25°C) for 30 min in the dark. The absorbance was taken at 515 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu) and expressed in percent free radical-scavenging activity calculated as Equation 2. Where, Abs_0 = Absorbance of control; Abs_1 = Absorbance of sample.

Equation 2:

$$\left(Abs_0 - \frac{Abs_1}{Abs_0} \right) \times 100\%$$

The scavenging ability of samples was expressed as IC_{50} value, reflecting the effective concentration at which 50% of DPPH radicals were scavenged. The IC_{50} values were calculated from the relationship curve of scavenging activities (%) versus concentrations of the sample. Trolox at different concentrations (10 µM to 800 µM) was used as the standard for antioxidants. The results were expressed as Trolox equivalents (TE), µM TE/g fresh mass. The analysis was done in minimum light exposure with triplicates.

Ferric reducing ability assay (FRAP)

The FRAP was done according to Benzie & Strain (1996) and Thaipong *et al*. (2006). FRAP reagents included 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl, 20 mM ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) solution and 300 mM acetate buffer pH 3.6 were freshly prepared and mixed as working solutions (warmed at 37°C before used). The fresh working solutions were prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solutions. A 150 µL of the crude ethanolic extracts (1 mg/mL) were allowed to react with 2850 µL of FRAP solution. Then, the crude ethanolic extracts were incubated for 30 min in the dark. The absorbance was taken at 593 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu) and expressed as µM TE/g extract. Trolox at different concentrations (10 µM to 800 µM) was used as the standard for antioxidants.

Ferrous ion chelating (FIC)

The FIC was determined according to the published method by Dinis *et al*. (1994) and Chew *et al*. (2009). A 1 mL of crude ethanolic extracts (1mg/mL) was mixed with 1 mL of 0.1 mM $FeSO_4$ and 1 mL of 0.25 mM ferrozine solutions. The mixture was mixed well with vortex and incubated for 10 min a room temperature (25°C). The absorbance was measured at 562 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu). The percentage of the chelating effect of diluted crude ethanolic extracts was calculated using the Equation 2.

Statistical analysis

All data in this study were first tested for their normality using the Shapiro-Wilk test. The data met the criteria for normality, allowing for the application of parametric tests. A one-way analysis of variance (ANOVA) was subsequently performed, followed by Tukey's post hoc test, with a significance level set at $P \leq 0.05$. All the analyses were conducted using statistical analysis software (SAS) Studio 3.8 (SAS Institute Inc., Cary, NC, USA., 2023). Pearson's correlation analyses were used to correlate colour, ΔE, pH value, TAC, TPC, TFC, DPPH inhibition, FRAP and FIC.

RESULTS AND DISCUSSION

Lower chromaticity value a* and higher value of chromaticity value b*, L* and h° indicate duller red colour of roselle decoction residues (Table 1). The DCR showed a similar colour to FRC, meanwhile, DJR showed duller in colour than DCR and FRC. Based on ΔE results, the colour difference between both FRC and roselle decoction residues (DCR and DJR) was perceptible at a glance indicated by the ΔE value of roselle decoction residues (ranges from 4.57 ± 0.98 to 5.07 ± 0.66) when compared to FRC. Based on pH analysis, The pH levels of roselle decoction residues (DCR and DJR) were still in acidic condition. The stability of anthocyanin colour depends on the pH value (Azza *et al*., 2011; Nur Amirah, 2015; Wahyuningsih *et al*., 2017). At lower pH, the anthocyanin's colour appears red and turns into blue colour as the pH increases (Khoo *et al*., 2017; Enaru *et al*., 2021). In an acidic environment, at pH 1, the anthocyanins are found in the form of flavylium cation (red colour). As the pH increases, the flavylium cations quickly hydrate and form colourless carbinol pseudobase between pH 4 and 6. In alkaline conditions, the dominant equilibrium is the acid-base equilibrium, leading to rapid proton transfer reactions and the formation of unstable products that degrade easily into other substances. The colours range from purple to violet as the solution's pH value changes from 6 to 8. These four different chemical forms of anthocyanins can also co-exist together at pH 2 - 4 with the presence of flavylium cation. At pH 7 and higher, anthocyanins exist as chalcone, resulting in a colourless solution. Under the same external conditions, higher pH values lead to faster degradation rates of anthocyanins. (Reyes & Cisneros-Zevallos, 2007; Castañeda-Ovando *et al*., 2009; Pina *et al*., 2012; Khoo *et al*., 2017; Kang *et al*., 2021; Enaru *et al*., 2021). In the present study, the FRC and roselle decoction residues were still in acidic conditions in the range of pH 2 to 4. The potential of these four different chemical forms of anthocyanins can also co-exist together with the presence of flavylium cation is possible as the roselle decoction residues still showed red.

Anthocyanins undergo colour alterations and degradation at varying pH levels, with acidic conditions favouring red hues and alkaline conditions inducing blue tones. Temperature variations also impact anthocyanin stability, with optimal ranges differing based on the source material. Notably, co-pigmentation, as elucidated by Gençdağ *et al*. (2022), emerges as a promising method to bolster anthocyanin stability. Through the formation of non-covalent complexes with natural co-pigments like polymers and phenolic compounds, anthocyanins can attain enhanced stability and colour modulation. The stability of anthocyanins and their colour in concentrated form, both as a food ingredient and in final products, is influenced by various factors (Gençdağ *et al*., 2022). These include food processing

and storage conditions, which can cause significant damage to anthocyanins due to their vulnerability to exogenous parameters like temperature, pH, oxygen, light, metal ions, enzymes, ascorbic acid, and sulphur dioxide (He *et al*., 2016; Liu *et al*., 2019). Enhancing anthocyanin stability is crucial for improving the quality of anthocyanin products. Different methods, such as encapsulation, copigmentation, and the use of metallic ions, have been explored to enhance anthocyanin stability. Copigmentation, which relies on intermolecular interactions like electrostatic, hydrogen bonding, and hydrophobic interactions, has proven to be particularly effective. This technique is commonly employed in the food industry to adjust food colour, retain or restore natural colour intensity, and create new hues. The addition of organic acids, flavonoids, alkaloids, polysaccharides, proteins, and amino acids has been shown to increase the stability and even modify the bioactivity of anthocyanins (He *et al*., 2016).

In addition to those factors, Contreras-Lopez *et al*. (2014) studied the influence of light on anthocyanin stability in ethanolic extracts and their research revealed a degradation pattern that followed second-order kinetics across varying light intensities. Notably, higher illuminance levels hastened anthocyanin degradation, underscoring the susceptibility of these compounds to light exposure. Similarly, Modesto Junior *et al*. (2023) examined the stability of anthocyanins under diverse temperature and light settings. Their findings demonstrated substantial degradation of anthocyanins under UV irradiation, whereas incandescent light exhibited superior stability. These observations highlight the critical role of light conditions in preserving anthocyanin content during both processing and storage.

Moreover, pH, temperature, and co-pigmentation are pivotal factors influencing anthocyanin stability (Khoo *et al*., 2017; Enaru *et al*., 2021). Despite these advancements, gaps in knowledge persist, particularly regarding the effects of light-sensitive compounds during extraction processes. The potential consequences of light exposure on anthocyanin stability during extraction warrant further investigation. Developing extraction methods that minimize light exposure while maximizing anthocyanin yield could address this gap and improve the efficiency of roselle-based products.

Table 1. Colour and pH value for roselle decoction residues

Sample	Colour						pH value
	L*	a*	b*	h°	C*	ΔE	
FRC	26.40 ± 0.68 ^{b}	19.05 ± 1.50 ^{a}	4.77 ± 0.75 ^{b}	13.98 ± 1.15 ^{c}	19.64 ± 1.63 ^{a}	0.00 ± 0.00 ^{b}	2.40 ± 0.01 ^{c}
DCR	22.66 ± 0.51 ^{c}	15.67 ± 1.43 ^{b}	4.83 ± 0.58 ^{b}	17.06 ± 0.82 ^{b}	16.44 ± 1.48 ^{b}	5.07 ± 0.66 ^{a}	2.80 ± 0.01 ^{b}
DJR	27.60 ± 0.49 ^{a}	14.86 ± 0.79 ^{b}	5.80 ± 0.30 ^{a}	21.34 ± 0.99 ^{a}	15.96 ± 0.80 ^{b}	4.57 ± 0.98 ^{a}	3.05 ± 0.01 ^{a}

[#]Note. Values are expressed as mean ± standard deviation (SD). Means with different letters are significantly different at the 5% level according to the Tukey test. L*: lightness, a*: chromaticity values a*, b*: chromaticity values b*, h°: hue, C*: chroma, ΔE: delta E, FRC: fresh roselle calyx, DCR: decocted cordial residue, DJR: decocted juice residue

In this study, the decoction process has significantly reduced the concentration of TAC as observed in DCR and DJR when compared to FRC (Table 2). The TAC in FRC (126.18 ± 3.76 mg/ 100 g FW) had the highest TAC value followed by DCR (73.34 ± 1.26 mg/100 g FW), and DJR (20.59 ± 0.78 mg/100 g FW). The decreased TAC values in DCR and DJR may be due to the effect of pH and colour stability of roselle residues itself. As the pH increases, the colour becomes duller resulting in lower anthocyanin content of DCR and DJR. This was supported by Wan Zaliha and Singh (2010a, 2010b) stated that the improvement in fruit colour coincided with the increase in TAC. The DCR showed a decrease of TAC up to 42% from FRC, meanwhile, DJR showed a larger decrease of TAC up to 84% when compared to FRC. The TPC and TFC were also summarized in Table 2. The FRC and DCR were found to have the same level of TPC, 175.45 ± 1.73 mg GAE/g extract to 185.01 ± 15.27 mg GAE/g extract, respectively, while DJR contained significantly low TPC, 75.24 ± 11.97 mg GAE/g extract. For the TFC assay, it was noticed that the value of TFC for DCR was comparable with FRC (98.13 ± 5.79 mg QE/g extract and 93.82 ± 3.28 mg QE/g extract, respectively). Meanwhile, the TFC value for DJR was significantly low (15.14 ± 15.17 mg QE/g extract). The reduction of Folin-Ciocalteu's reagent by electron transfer during phenol oxidation in alkaline conditions is obtained by the addition of sodium carbonate in the TPC assay. The intensity of the blue colour reflects the quantity of phenolic compounds by using a spectrophotometer (Rohaya & Noriham, 2013). The degree of colour change was correlated to the concentration of antioxidants in the sample.

From the TAC, TPC and TFC results obtained, it can be seen that the roselle decoction residues, mainly DCR, still possess a valuable source of anthocyanins, TPC and TFC. This may be due to the activity of phenolic and flavonoid compounds present in the extract. These obtained residue extracts can be potentially used in the food and pharmaceutical industries. Numerous researchers also have emphasized the functional properties of roselle and its extracts, which offer opportunities for the development of novel products with enhanced nutritional characteristics. Apart from contributing to the

food colourant, anthocyanins, and other phenolic compounds are well-documented in reducing chronic diseases such as various cancers, particularly lung, prostate, liver and colon cancer, cardiovascular diseases, asthma, diabetes mellitus, dyslipidemia, and hypertension. (Boyer & Liu, 2004; Cid-Ortega & Guerrero-Beltrán, 2015). These polyphenols are very strong antioxidants, that act as anti-oxidative, anti-mutagenic, anti-microbial, and anti-carcinogenic (Awad *et al*., 2000; Lachowicz *et al*., 2019) which inhibits cancer cell proliferation, decreases lipid oxidation and lowers cholesterol.

Table 2. TAC, TPC, and TFC of roselle decoction residues

Sample	TAC (mg/100 g FW)	TPC (mg GAE/g extract)	TFC (mg QE/g extract)
FRC	126.18 ± 3.76 [a]	175.45 ± 1.73 [a]	93.82 ± 3.28 [a]
DCR	73.34 ± 1.26 [b]	185.01 ± 15.27 [a]	98.13 ± 5.79 [a]
DJR	20.59 ± 0.78 [c]	75.24 ± 11.97 [b]	15.14 ± 15.17 [b]

[#]Note. Values are expressed as mean ± standard deviation (SD). Means with different letters are significantly different at the 5% level according to the Tukey test. TAC: total anthocyanin concentration, FW: fresh weight, TPC: total phenol content; GAE: gallic acid equivalents, TFC: total flavonoids content; QE: quercetin equivalents, FRC: fresh roselle calyx, DCR: decocted cordial residue, DJR: decocted juice residue

Table 3 represents the DPPH radical scavenging abilities and inhibition concentration (IC_{50}) of roselle decoction residues. The higher the percentage of DPPH radicals scavenging activity, the better the antioxidant activity. As expected, a high percentage of DPPH was observed in FRC (20.57 ± 1.79%). The DJR resulted in the lowest value of DPPH inhibition (2.61 ± 0.79%), followed by DCR (19.88 ± 1.29%) in ascending order. The data showed that they have noticeable antioxidant activities present that can be attributed to an anthocyanin content in FRC and roselle decoction residues (DCR and DJR).

The concentration required to inhibit the 50% free radical-scavenging effect (IC_{50}) has also been measured using a wide range of extract concentrations. Unlike the DPPH assay, a lower IC_{50} value indicates a greater scavenging activity, and vice versa. The FRC significantly showed the lowest values of IC_{50}, which was 7.83 ± 1.66 µg/mL. Meanwhile, DCR also had lower IC_{50} values (9.00 ± 2.51 µg/mL) when compared to DJR (41.06 ± 3.57 µg/mL).

DPPH is a stable free radical due to the delocalization of the spare electron throughout the molecule, which prevents the molecules from dimerizing like most other free radicals. This delocalization produces a deep purple colour and then turns yellow as the odd electron/radical of the DPPH radical readily accepts an electron or hydrogen radical to become a stable diamagnetic molecule, thereby neutralizing its free radical character (Molyneux, 2004; Rohaya & Noriham, 2013; Amin *et al*., 2013). In other words, the higher the percentage of DPPH radicals scavenging activity, the better the antioxidant activity. The data in the present study agreed with Sharara (2017) and Ajiboye *et al*. (2011). They found that the roselle calyx has a high antioxidant content due to the presence of anthocyanin, which has strong antioxidant properties. Therefore, from the result obtained, it is understood that the ability of DCR in scavenging activity was higher compared to the DJR. No significant effect was recorded on the DPPH radical scavenging activity as compared between FRC and DCR. Hence, DCR showed better antioxidant activity similar to control or FRC.

For FRAP, the DCR exhibited good ferric ion-reducing antioxidant power (209.22 ± 20.43 µM TE/g extract) exhibiting comparable to the FRC (243.55 ± 6.88 µM TE/g extract), while DJR possessed very low FRAP activity with values of 68.44 ± 2.82 µM TE/g extract (Table 3). In the FRAP assays, the reducing power of a compound was evaluated by direct electron donation from the antioxidant to the ferric (Fe3+) ion complex. This chemical reaction involved would cause the reduction of this ion complex into the ferrous (Fe2+) ion complex. The more ferric ions are reduced to ferrous ions by an antioxidant, the more intense the blue colour formation is perceived (Rohaya & Noriham, 2013; Amin *et al*., 2013). In other words, the higher the FRAP values, the higher the antioxidant activity obtained. Therefore, DCR in the present study showed good antioxidant activity as the FRAP values remained higher and similar value ranges to FRC. In contrast, DJR had lower antioxidant activity.

The FRC was found to have the best chelating effect on ferrous ion (63.91 ± 0.25%), and the lowest in DJR (10.43 ± 2.90%) as illustrated in Table 3. The FRC had the highest ferrous ion chelating activity followed by DCR indicating that the extract interfered with the formation of the metal-antioxidant complex and captured the ferrous ion first before ferrozine. When chelating agents are present, they will inhibit the formation of ferrozine metal ion complexes, hence decreasing the intensity of the red colour (Heijnen *et al*., 2002; Qaid *et al*., 2014; Gulcin & Alwasel, 2022). Secondary metabolites present in roselle decoction residues may be responsible for increasing the metal-chelating activity of extracts. The significant effect of the metal chelating activity is to slow down lipid peroxidation by reducing the concentration of transition metal ions. Chelating agents stabilized the metal ion's oxidized state by lowering its redox potential (Md Yusof *et al*., 2013; Kalin *et al*., 2015; Gulcin & Alwasel, 2022). The

antioxidant compounds contained in the DJR extract probably can also inhibit the interaction between metal and lipid peroxidation through the formation of insoluble complexes with ferrous ions, even though the DJR had the lowest chelating effect on ferrous ions. Therefore, the FRC and roselle decoction residues (DCR & DJR) may contain a secondary metabolite that can increase the chelating activity and slow down the lipid peroxidation process.

Previous studies have identified polyphenolic compounds such as anthocyanins, flavonoids, quercetin, kaempferol, cyanidin, and phenolic acids in roselle, known for their strong chelating abilities and antioxidant properties (Kori *et al*. 2020; Negreanu-Pirjol *et al*., 2023). Among these, cyanidin-3-sambubioside and delphinidin-3-sambubioside are prominent anthocyanins found in roselle that exhibit potent metal chelating activity (Peredo Pozos *et al*., 2020). Additionally, organic acids such as citric acid and tartaric acid present in roselle may contribute to its antioxidant and metal-chelating activities. Citric acid, for instance, has been reported to enhance iron chelation and inhibit lipid peroxidation. Therefore, the presence of these specific metabolites in roselle decoction residues likely enhances their chelating activity and antioxidative potential, aligning with the observed findings in this study.

The primary antioxidants scavenge free radicals and interrupt chain initiation and propagation, preventing the formation of new chains, meanwhile, secondary antioxidants suppress the formation of free radicals and protect against oxidative damage (Lim *et al*., 2007; Lobo *et al*., 2010). Based on those antioxidant analyses, there is a noticeable difference in antioxidant activities between DCR and DJR. The results showed that FRC may possess potent primary and secondary antioxidant properties. Meanwhile, the DCR may act as moderate primary and secondary antioxidant properties. Despite having lower TPC, TFC, and DPPH inhibition values, DJR may potentially become potent secondary antioxidants because they contain active components that can bind to metal ions. This would be an interesting point to note and should warrant further investigation.

Table 3. Inhibition activity of DPPH radical scavenging, IC_{50} value, FRAP, and FIC of roselle decoction residues

Sample	DPPH inhibition (%)	IC_{50}	FRAP (μM TE/g extract)	FIC (%)
FRC	20.57 ± 1.79 [a]	7.83 ± 1.66 [b]	243.55 ± 6.88 [a]	63.91 ± 0.25 [a]
DCR	19.88 ± 1.29 [a]	9.00 ± 2.51 [b]	209.22 ± 20.4[3 b]	38.37 ± 1.18 [b]
DJR	2.61 ± 0.79 [b]	41.06 ± 3.57 [a]	68.44 ± 2.82 [c]	10.43 ± 2.90 [c]

[#]Note. Values are expressed as mean ± standard deviation (SD). Means with different letters are significantly different at the 5% level according to the Tukey test. FRAP: ferric ion reducing antioxidant power, TE: trolox equivalents, FIC: ferrous ion chelating, FRC: fresh roselle calyx, DCR: decocted cordial residue, DJR: decocted juice residue

Correlation

The Pearson correlation coefficients between colour, ΔE, pH value, TAC, TPC, TFC, DPPH, FRAP, and FIC provide valuable insights into the relationships among these variables in roselle decoction residues. The correlation coefficient analysis between all the analyses conducted in this study is presented in Table 4. The correlation analysis showed there are strong positive correlations between various colour parameters (L*, a*, b*) and some antioxidant activities (DPPH inhibition, FRAP, FIC), indicating that certain colour attributes might be indicative of antioxidant potential. Interestingly, the negative correlations between colour parameters and antioxidant activities might indicate complex interactions between colour compounds and antioxidant compounds, possibly suggesting that certain colour compounds could interfere with antioxidant mechanisms or act synergistically with them.

There was a positive relationship between pH value and colour indicating a strong relation between them (Nur Amirah, 2015; Wu *et al*., 2018; So *et al*., 2021). Interestingly, TAC had a strong correlation with colour attributes of a* and b* values (r=.805 and r= −.585, respectively) while TAC, TPC, TFC, DPPH and FRAP were highly correlated to h° (r= −.946, r= −.833, r= −.832, r= −.865, and r= −.918, respectively) of the samples. Although the antioxidant content and activities did not have a direct correlation with the pH value, they were indirectly correlated via colour attributes. Moreover, the strong negative correlation between pH value and antioxidant activities suggests that acidity might enhance the antioxidant potential of the residues, likely due to the stabilization of bioactive compounds under acidic conditions.

Additionally, significant positive correlations exist between TAC, TPC, TFC, and antioxidant activities, supporting the notion that these bioactive compounds contribute significantly to the antioxidant capacity of roselle decoction residues. The highest positive correlation was recorded between DPPH and FRAP (r=.979), followed by TPC, TFC, and TAC (r=.975, r=.970, and r=.876, respectively). The highest correlation between DPPH and FRAP may be due to the similar mechanisms involved, in which the ability of antioxidants to reduce radicals. Furthermore, the correlations between TPC, TFC, and antioxidant activities are particularly noteworthy, indicating that phenolic and flavonoid compounds play

Table 4. Pearson's Correlation coefficients between colour, ΔE, pH value, TAC, TPC and TFC, DPPH inhibition, FRAP and FIC

	L*	a*	b*	h°	C*	ΔE	pH value	TAC	TPC	TFC	DPPH inhibition	FRAP	FIC
L*	1	.128	.499	.319	.180	−.345	.106	−.223	−.703*	−.670*	−.631*	−.509	.749**
		.650	.059	.247	.520	.208	.708	.424	.004	.006	.012	.053	.001
a*	.128	1	−.039	−.675*	.995***	−.792**	−.824**	.805**	.495	.518*	.577*	.684*	.713*
	.650		.890	.006	<.0001	.000	.000	.000	.061	.048	.024	.005	.003
b*	.499	-.039	1	.760**	.054	.301	.557*	−.585*	−.665*	−.644*	−.647*	−.632*	−.507*
	.059	.890		.001	.848	.276	.031	.022	.007	.010	.009	.012	.054
h°	.319	−.675*	.760**	1	−.604*	.708*	.932***	−.946***	−.833**	−.832**	−.865***	−.918***	.669*
	.247	.006	.001		.017	.003	<.0001	<.0001	.000	.000	<.0001	<.0001	.006
C*	.180	.995***	.054	−.604*	1	−.767**	−.772**	.749**	.431	.456	.512*	.621*	.842***
	.520	<.0001	.848	.017		.001	.001	.001	.109	.087	.051	.013	<.0001
ΔE	−.345	−.792**	.301	.708*	−.767**	1	.853***	−.792**	−.323	−.322	−.431	−.555*	−.441
	.208	.000	.276	.003	.001		<.0001	.000	.241	.241	.109	.032	.100
pH value	.106	−.824**	.557*	.932***	−.772**	.853***	1	−.992***	−.740*	−.750**	−.812**	−.888***	.346
	.708	.000	.031	<.0001	.001	<.0001		<.0001	.002	.001	.000	<.0001	.207
TAC	−.223	.805**	−.585*	−.946***	.749**	−.792**	−.992***	1	.814**	.819**	.876***	.935***	−.985***
	.424	.000	.022	<.0001	.001	.000	<.0001		.000	.000	<.0001	<.0001	<.0001
TPC	−.703*	.495	−.665*	−.833**	.431	−.323	−.740*	.814**	1	.986***	.975***	.942***	−.969***
	.004	.061	.007	.000	.109	.241	.002	.000		<.0001	<.0001	<.0001	<.0001
TFC	−.670*	.518*	−.644*	−.832**	.456	−.322	−.750**	.819**	.986***	1	.970***	.947***	−.983***
	.006	.048	.010	.000	.087	.241	.001	.000	<.0001		<.0001	<.0001	<.0001
DPPH inhibition	−.631*	.577*	−.647*	−.865***	.512*	−.431	−.812**	.876***	.975***	.970***	1	.979***	−.961***
	.012	.024	.009	<.0001	.051	.109	.000	<.0001	<.0001	<.0001		<.0001	<.0001
FRAP	−.509	.684*	−.632*	−.918***	.621*	−.555*	−.888***	.935***	.942***	.947***	.979***	1	−.824**
	.053	.005	.012	<.0001	.013	.032	<.0001	<.0001	<.0001	<.0001	<.0001		0.000
FIC	.749**	.713*	−.507*	.669*	.842***	−.441	.346	−.985***	−.969***	−.983***	−.961***	−.824**	1
	.001	.003	.054	.006	<.0001	.100	.207	<.0001	<.0001	<.0001	<.0001	0.000	

#Note. *** correlation is significant at the $p<0.0001$ level (2-tailed); ** correlation is significant at the $p<.001$ level (2-tailed); * correlation is significant at the $p<.05$ level (2-tailed). L*: lightness, a*: chromaticity values a*, b*: chromaticity values b*, h°: hue, C*: chroma, ΔE: delta E, TAC: Total anthocyanin concentration, TPC: total phenol content, TFC: total flavonoids content, FRAP: ferric ion reducing antioxidant power, FIC: ferrous ion chelating

pivotal roles in the observed antioxidant properties. The strong positive correlations between DPPH inhibition, FRAP, FIC, and TAC further emphasize the importance of these bioactive compounds in scavenging free radicals and exhibiting reducing and chelating abilities. Present results also revealed that flavonoids are an important phenolic group in representing the antioxidant capacity of FRC. In fact, from the result obtained, the excellent correlation proved that the phenolic compounds are the main constituent contributing to the antioxidant activities of FRC and roselle decoction residues. A strong correlation has also been reported in various studies on roselle calyx (Kouakou *et al*., 2015; Sharara, 2017; Mohd Ali *et al*., 2019). Overall, these correlation analyses shed light on the intricate relationships between colour attributes, biochemical compositions, and antioxidant activities in roselle decoction residues, providing valuable insights for understanding their potential health benefits and applications in food and beverage industries.

Exploring potential research arising from the correlation analysis of colour attributes, biochemical compositions, and antioxidant activities in roselle decoction residues offers promising directions for further investigation. Future studies could delve into elucidating the underlying mechanisms driving these correlations, shedding light on how colour compounds interact with antioxidants and the influence of pH on such interactions. Additionally, optimizing processing techniques to retain bioactive compounds and antioxidants could be a focus, considering the impact of extraction methods, temperatures, and durations on both colour profiles and antioxidant potentials. Understanding the bioavailability of these compounds and their effects on health, alongside exploring their application in functional foods, presents avenues for future exploration. Clinical trials could further validate their efficacy in managing oxidative stress-related conditions, potentially broadening their therapeutic applications. Pursuing these avenues could deepen our comprehension of the observed correlations and unlock the diverse potential of roselle decoction residues across various industries.

CONCLUSION

From the results obtained, the roselle decoction residues did have differences in the presence of valuable compounds between the roselle decoction residues and control, thus rejecting the null hypothesis. This study has been successful in giving a clearer view of the antioxidant content and activities of roselle decoction residues (DCR & DJR). Between the DCR and DJR, the DCR showed promising results in both antioxidant content and activity. The DCR exhibited comparable or slightly lower antioxidant content and activity compared to the FRC, with values for TAC, TPC, TFC, DPPH, FRAP, and FIC at 73.34 ± 1.26 mg/100 g FW, 185.01 ± 15.27 mg GAE/g extract, 98.13 ± 5.79 mg QE/g extract, 19.88 ± 1.29%, 209.22 ± 20.43 µM TE/g extract, and 38.37 ± 1.18%, respectively, but notably outperformed the DJR. Hence, it can be concluded that DCR can be turned into a valuable resource as an alternative source of natural antioxidants. More research is necessary to uncover its potential uses in functional foods, supplements, and pharmaceuticals. Utilizing roselle decoction residues can promote a greener, more sustainable approach to food and beverage manufacturing.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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