Quantification of polyphenol, antioxidant, and antibacterial from red and purple roselle calyces using maceration extraction under different solvent conditions

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Abstract. This research was conducted to extract polyphenols, antioxidants, and antimicrobial red and purple roselle calyces (Hibiscus sabdariffa L.) with maceration techniques using different types of solvents. Total polyphenols were determined using the Folin-Ciocalteu method. The antioxidant activity was expressed as the DPPH reduction, and antimicrobial activity were evaluated against the Staphylococcus aureus and Escherichia coli bacterial strains. The most significant extraction yield was obtained using ethanol at a concentration level of 80% in two rosella varieties (red and purple). In contrast, the polyphenol content of purple roselle was relatively higher than that of red roselle. The maximum total polyphenols in the extraction process in purple roselle with methanol 80% (27.60 mg GAE/g), while the minimum phenolic (9.50 mg GAE/g extract) in red roselle with 50% ethyl acetate solvent. The antioxidant activity increased in the antioxidant test, where 80% methanol extract showed the highest scavenging antioxidant activity, 73.84 ± 0.53% and 62.80 ± 1.57% for purple and red roselle calyces. The inhibition zone was obtained against E. Coli (13.45 ± 3.30 mm) on the purple roselle, and the inhibition zone was obtained for S. aureus (11.4 ± 0.04) on the red roselle. It was concluded that red and purple roselle calyces are a prospective source of antioxidant and antimicrobial phenolic compounds.

1. Introduction
Roselle (Hibiscus Sabdariffa L.) has been known as a fiber-producing plant, and it contains secondary metabolite bioactive compounds. Roselle calyces are an important part that is rich in vitamins, minerals, and bioactive content such as organic compounds, phytosterols, and polyphenols, and anthocyanins can act as antioxidants [1-2]. Roselle calyces are known to contain saponins, flavonoids, and polyphenolics which have medicinal properties related to active chemical components found in these plants, especially phytochemical compounds [3-4]. Physiological and pharmacological beneficial effects of roselle petals include antioxidant, antibacterial, antifungal, anti-inflammatory, antidiabetic, and antihypertensive activity [5–7]. The phenolic compounds in roselle flower petals are strongly suspected as compounds that play an important role in the antioxidant and antibacterial activity of rosella calyces.

There are 2 types of roselle, red and purple roselle, in Indonesia. The bioactive compounds possessed by red and purple roselle petals are obtained through an extraction process. One of the extraction processes that are often used is by using a solvent. The organic solvent selection method is very important to determine the success of the extraction. Based on the dissolves, such as solubility...
law, the solvent's polarity must be close to the polarity of the extracted substance [8]. The extraction method with solvents is often applied at the beginning of the preparation of extracts of plant simplicia because its application is more efficient, broad, and easy to use. Ethanol, methanol, acetone, and ethyl acetate are some of the most common kinds of organic solvents used for the extraction of phenolic compounds.

Several researchers have reported solvent extraction of rosella using water and ethanol [9], acidified methanol [10]. The varying polarity of plant bioactive compounds and solvents causes differences in the number and activity of bioactive compounds contained therein. However, no researcher has taken steps to compare the different types of solvents and different concentration levels used to extract red and purple roselle and total phenolic, antioxidant, and antibacterial activity. Maceration is one of the conventional methods (CSE) that is considered to use a large amount of solvent and relatively longer extraction time so that it is relatively low efficiency. However, the maceration extraction method is also easy to do, environmentally friendly, and requires relatively low cost [11]. The research results on polyphenols by Safdar et al. [12] on kinnow skins show that the maceration extraction method using 80% ethanol solvent can produce greater extraction yields than with other organic solvents.

Plants produce secondary metabolites as their defense system during growth. The secondary metabolite phenolic compounds can inhibit the activity of microorganisms such as fungi and bacteria [13]. Some research on the antimicrobial activity of plant phenolic groups has been carried out, including Rahayu et al. [14], which stated that plant phenolic compounds were shown to have antibacterial activity inhibit Staphylococcus aureus, Staphylococcus sp., and Escherichia coli bacteria. Based on the facts detailed above, this experimental research study is designed to maximize the extraction yield for polyphenols for red and purple roselle petal polyphenols and establish the antioxidant and antimicrobial function of phenolic compounds in roselle calyces.

2. Methods

2.1 Plant material

Red and purple rosella calyces (Hibiscus sabdariffa L) were purchased from Roselle Garden in Malang, East Java, Indonesia. Roselle flowers are then washed thoroughly under tap water to remove dust and dirt. The rosella flowers are then chopped into small pieces using a knife and dried in a cabinet dryer at 50°C in a hot air oven for 36 hours before the moisture content reaches 9-10%. The dried rosella calyces are then ground to become powdered rosella calyces with a mesh size of 60.

2.2 Maceration extraction

Roselle calyces powder was exposed to extraction by maceration extraction, as illustrated by Sanhueza et al. [15]. With minor alterations. The extraction process is carried out by maceration using methanol, ethanol, and ethyl acetate solvents at two solvent levels (50% and 80%) with a sample/solvent ratio of 1:10 at room temperature. 100 g of rosella flower petal powder was immersed in hexane, ethyl acetate, and 70% ethanol, respectively, with a ratio of 1:10 (w/v). The following process is macerated for 24 hours at room temperature (25 °C) while shaking with a shaker. The extract was then filtered with Whatman 1 filter paper and centrifuged (Shimadzu, model HC1180T) at 1000 rpm for 15 minutes, then evaporated by a rotary evaporator under vacuum at 45 °C to obtain clear supernatant. The extract was then filtered through a membrane filter of 0.5 mm, poured into a glass bottle, and kept at a cooling temperature.

2.3 Total polyphenol determination

Total polyphenol levels of red and purple roselle flower extracts were determined using the Foline Ciocalteau method described by Blainski et al. with slight modification [16]. In short, a 0.4 ml extract sample solution was then mixed with 1.5 ml of FolinCiocalteau reagent solution (10% v/v) and mixed with 1.5 ml 7.5% (w/v) Na2CO3 solution. Then, the samples were incubated for 30 minutes at room
temperature (25 °C) and dark to see the progress showed a blue color change. After that, the absorbance measurement was measured by Spectrophotometry UV-Vis (Shimadzu, model 1800) at an absorbance of 765 nm. A similar procedure was performed for gallic acid standards, and a calibration curve was prepared from different gallic acid concentrations. The total content of polyphenols was described as mg gallic acid equivalent (GAE)/g extract equivalent.

2.4 Antioxidant activity evaluation

The antioxidant activity test of radical scavenging in roselle flower extract was carried out by the scavenging activity method on DPPH using spectrophotometry based on Sun et al. with slight modification [17]. In short, we were making DPPH solution (1 mg ml\(^{-1}\)) dissolving 50 mg DPPH in 50 ml methanol for the supply of solutions. Rosella flower extract was diluted again in methanol (1 mg ml\(^{-1}\)) at the difference in levels (25, 50, 100, 200, and 400 ppm). Each concentration of 2 ml extract was added with 2 ml of DPPH solution, then shaken until homogeneous and incubated in a dark room for 30 minutes. The light absorption by the solution was measured at a wavelength of 517 nm using spectrophotometry. The DPPH solution without sample and a standard mark was used as control. Ascorbic acid was also analyzed and used as a standard curve. DPPH reduction was expressed as % inhibition of DPPH radicals.

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\text{% Radical DPPH inhibition} \% = \frac{A_C - A_S}{A_C} \times 100
\]

Where the monitoring absorbance is \(A_C\), and the sample absorbance is \(A_S\). The amount of antioxidant activity is indicated by the value \(IC_{50}\) [13].

2.5 Antimicrobial activity determination

The antimicrobial activity of roselle flower extract was determined using the disc dilution method [18]. Two strains of tested bacteria used were \textit{Staphylococcus aureus} ATCC 25923 and \textit{Escherichia coli} ATCC 25922. For the manufacture of nutrient agar (NA), solid media in Water was purified, and the pH was changed to 7. Media sterilization was carried out for 15 minutes in an autoclave at 120, and the media was cooled after sterilization to the temperature room. For further investigation, Petri dishes without contamination were chosen. On agar media, sterile paper discs measuring 6 mm in diameter are positioned. A total of 1 ml of sample ethanol extract with concentrations (500 and 1000 ppm) was applied to disc paper and then placed on a petri dish containing the medium to make it solid. Furthermore, the plates were incubated for 24 hours at 37 °C. Observations are made by measuring the clear zone around the disc paper with a capillary device that states the antibacterial activity.

2.6 Statistical analysis

Statistical data analysis used analysis of variance to determine the level of significance. To calculate the smallest significant difference between treatments, the minimum square design (LSD) test was used. The Program from Minitab is used to conduct statistical data analysis.

3. Results and discussion

3.1 Total polyphenol content

The maceration method was applied to extract polyphenols, which was measured using the FolinCiocalteau test reagent. Total polyphenol levels of red and purple roselle flower extracts showed that the extraction of purple roselle polyphenols using maceration techniques (Figures 1 and 2) was relatively higher than that of red roselle. The most powerful solvent followed by ethanol was methanol maceration extraction, while ethyl acetate displayed the lowest extraction rate for polyphenols. The largest overall content of polyphenols (27.60 ± 0.3 mg GAE/g) in purple rosella with methanol solvent with a concentration of 80% and the lowest polyphenols (9.50 ± 0.2 mg GAE/g extract) in red roselle with 50% ethyl acetate solvent. The outcome of the LSD test indicates that the amount of the solvent was a significant difference between the concentrations of 50% and 80% than the ethyl acetate solvent.
In extraction, the level of the polarity of the solvent plays a very important role. Solvent polarity is determined by the solvent concentration, solvent properties, and dielectric constant [19]. Dailey et al. [20] stated that the dielectric constants of methanol, ethanol, and ethyl acetate are as follows: 32.61; 24.30; 1.89.

![Figure 1. Total polyphenol (%) of rosella calyces red extract by maceration extraction. The letters of the same alphabet indicate nonsignificant at p < 0.05.](image1)

![Figure 2. Total polyphenol (%) of rosella calyces purple extract by maceration extraction. The letters of the same alphabet indicate at p < 0.05.](image2)

In this research, the methanol solvent had the highest polarity level compared to others, resulting in the largest polyphenol content followed by ethanol solvent. In research by Naseer et al. [21] describing the extraction of polyphenols in roselle calyces using 80% methanol solvent for 2 hours at room temperature, rosella flower petals contain phenolic compounds of 2.91 mg GAE/g greater than water solvent of 1.85 mg GAE/g. In general, the extraction of phenolic compounds from plant
Simplicia, alcohol forms such as methanol and ethanol are strong organic solvents. [22]. Methanol is well used to extract polyphenol compounds because of its lighter molecular weight [23]. Ethanol is categorized as the preferred and safe solvent due to its application in functional food systems. Ethanol can increase the solubility of solutes in phenolic compounds, and there may be a strong hydrogen bond between polyphenols and proteins [24].

3.2 Antioxidant activity
DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical compound. In his experiments, the DPPH solution will change from a dark purple color to light yellow [25]. In this research, antioxidant activity analysis was carried out using methanol and ethanol solvents with the largest extract and polyphenol yields. This is done because, based on existing research, samples with high extract and total polyphenols will produce high antioxidant activity [20]. The effect of different solvents and their concentration levels on the scavenging activity of DPPH radicals from red and purple roselle petal extracts (Table 1) showed high antioxidant activity of all extract samples. However, the scavenging activity was highest (73.84 ± 0.53%) in purple roselle and (62.80 ± 1.57) on red roselle calyces with 80% methanol extraction, and followed by ethanol extract samples (67.65 ± 0.34%), while the sample extracted with 50% ethanol had the lowest scavenging activity (52.45 ± 0.67%). The solvent extract with a mixture of water has a fairly large inhibitory power against DPPH radicals compared to pure solvents [16].

| Table 1. Antioxidant activity of red and purple roselle calyces extract |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| DPPH assay      | Methanol 80%    | 50%             | Ethanol 80%     | 50%             |
| Red roselle     | 62.80 ± 1.57a   | 54.80 ± 1.11c   | 59.28 ± 1.63b   | 52.45 ± 0.67d   |
| Purple roselle  | 73.84 ± 0.53a   | 63.67 ± 0.83c   | 67.65 ± 0.34b   | 58.06 ± 0.92d   |

Note: The letters of the same alphabet indicate nonsignificant at p < 0.05. All values reflect the mean ± standard error of three replications.

Samples extracted by solvent methanol and ethanol in the purple roselle showed greater antioxidant activity than the red roselle. Related research by Aryani et al. describes the extraction of flavonoid group anthocyanins which function as antioxidants with solvent extraction of water and ethanol with maceration techniques on purple roselle showing greater results (484.5 mg/L extracts) than red roselle (296, 8 mg/l extracts). The research of natural antioxidants by Do et al. [26] observed that 80% ethanol extract had scavenging activity of DPPH radicals compared to 50% of Limnophila aromatic leaves. IC₅₀ has a negative correlation value with antioxidants' activity, and the lower the IC₅₀ value indicates the greater the activity of antioxidants of the sample [27]. The analysis showed that 80% and 50% methanol solvent extracts had IC₅₀ of 440.64 ppm and 482.38 ppm, respectively, in purple roselle. The results also indicated that each extract's antioxidant activity was associated with total polyphenols, as conducted by Liew et al. [28].

3.3 Antimicrobial activity
Based on the data, the inhibitory activity of bacterial growth also increased with a higher concentration of roselle flowers, which implied that microbial growth was concentration dependent. Antibacterial activity is expressed by the length of the clear zone generated around the disc. Diameter < 6 mm indicates inactive extract while diameter > 6 mm, clarified extract has antibacterial activity [15]. Table 3 presents the antibacterial activity of ethanol extract in cases at a concentration of 1000 ppm/disk. The zone of inhibition (13.45 ± 3.30 mm) was recorded against E. coli in purple roselle. The inhibition zone was (7.80 ± 2.20 mm) at a concentration of 500 ppm against S. aureus on red roselle. The
analysis showed a significant difference between the two extract concentrations of the two strains of bacteria.

**Table 2. Antimicrobial activity of red and purple roselle calyces extract**

| Sample          | Extracts     | Conc. µg/ml | Inhibition Zone (mm) |  |
|-----------------|--------------|-------------|----------------------|--|
| Red roselle     | Ethanol 80%  | 500         | 7.80 ± 2.20<sup>a</sup> |  |
|                 |              | 1000        | 10.53 ± 1.05<sup>b</sup> |  |
| Purple roselle  | Ethanol 80%  | 500         | 8.96 ± 2.20<sup>c</sup>  |  |
|                 |              | 1000        | 12.50 ± 1.14<sup>c</sup> |  |

Note: The letters of the same alphabet indicate nonsignificant at p < 0.05. All values reflect the mean ± standard error of three replications.

The bioactive compounds involved can function as antimicrobials from plant extracts which have been extensively studied [29]. The phenolic compounds' quality as a stimulus to microorganism infection by plants is caused by the thickening of the bacterial cell membrane. The number of hydroxyl groups in polyphenol compounds is mainly affected by the antibacterial test. It can interfere with the bacterial cell wall to disrupt the membrane structure and cause leakage of cellular components. In this research, 80% ethanol extract against antibacterial activity resulted in a zone of inhibition against E. coli greater than S. aureus. This is also consistent with the research of Kumar *et al.* [30]. They reported that Aloe vera leaf extract could have more antibacterial activity, which is greater against E. coli than S. aureus at a concentration of 250 ppm.

4. Conclusion

*Roselle calyces* are rich in phenolic compounds and have high antioxidant activity. The maceration technique of purple roselle extract produced higher polyphenols than the red roselle. Efficient solvents for polyphenol extraction are ethanol solvents categorized as safer and preferred because of their application in the food system. Phenolic totals have a strong correlation with the antioxidant activity investigated. Regarding the antimicrobial test of red and purple roselle calyces extracts against two strains of foodborne bacteria, the zone of inhibition of ethanol extract can produce a zone of inhibition against E. coli greater than S. aureus. It was concluded that roselle calyces have a phenolic compound as a potential source of antioxidant and antimicrobial activity. As an ingredient for making functional foods, it can be used.

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