Comparison of flavonoid content and antioxidant activity in calyces of two roselle varieties (*Hibiscus sabdariffa* L.)

Comparison of flavonoid content and antioxidant activity in calyces of two roselle varieties (*Hibiscus sabdariffa* L.)

N Nerdy[1*], B R Barus[1], H J El-Matury[2], S Ginting[2], N F Zebua[3], T K Bakri[4]

[1]Department of Pharmacy, Institut Kesehatan Deli Husada Deli Tua, Sumatera Utara, Indonesia

[2]Department of Public Health, Institut Kesehatan Deli Husada Deli Tua, Sumatera Utara, Indonesia

[3]Department of Pharmacy, Universitas Tjut Nyak Dhien, Sumatera Utara, Indonesia

[4]Department of Pharmacy, Universitas Syiah Kuala, Aceh, Indonesia

*Corresponding author: nerdy190690@gmail.com

Abstract. Free radicals can cause various degenerative diseases such as cancer and cardiovascular disease. Flavonoids have antioxidant abilities that inhibit damage from free radicals. The roselle antioxidant mechanism is associated with high flavonoid content. Red and purple roselles are two varieties of roselle (*Hibiscus sabdariffa* L.) cultivated in Indonesia. The aim of this research was to analyze the differences in flavonoid content and antioxidant activity in the calyces of two varieties of roselle (*Hibiscus sabdariffa* L.), red and purple. Total flavonoid analysis and antioxidant activity testing were done by spectrophotometry in the visible region. Total flavonoid content was analyzed by pretreatment with aluminium chloride and sodium acetate. Antioxidant activity was tested with 2,2-diphenyl-1-picrylhydrazyl. The total flavonoids for both red and purple roselle calyces ethanolic extract were 27.55 and 39.18 mg quercetin equivalent per gram. The half maximal inhibitory concentration values obtained for both red and purple roselle calyces ethanolic extract a were 51.59 and 42.17 µg/mL. The total flavonoid content and antioxidant activity of purple roselle calyces ethanolic extract was higher and stronger than red roselle calyces ethanolic extract.

1. Introduction

Free radicals are molecules that contain one or more unpaired electrons. Free radicals are highly reactive and result in cross-linked deoxyribonucleic acids, proteins, and lipids, or oxidative damage to important functional groups in these biomolecules [1]. Excessive free radicals can trigger various degenerative diseases, such as cancer and cardiovascular disease [2]. Antioxidants are able to remove, clean, or neutralize the harmful effects of free radicals. Antioxidant compounds can donate one or more electrons to free radicals while the compound remains non-reactive and stable [3].

Several plant extracts have antioxidant compounds such as flavonoids which are more effective and safer than synthetic antioxidants, such as butylated hydroxytoluene [4]. Flavonoids inhibit peroxide radicals, hydroperoxides radicals, and oxidative mechanisms, and prevent degenerative diseases. Itfunctions as an antitumor and anticancer, as well as a hepatoprotective and nephroprotective [5].

Flavonoids have antioxidant abilities that have been shown to inhibit oxidative stress in cardiovascular and neurodegenerative diseases [6].

The high benefit of the herbal roselle (*Hibiscus sabdariffa* L.) plant is due to the natural phytochemicals found in all parts of the plant, especially in its petals, also known as calyces or flos. The active compound that acts as an antioxidant in roselle petals is flavonoid [7]. Roselle petals have been widely consumed in healthy drinks. Consuming roselle tea is associated with an increase in flavonoid content. There are two varieties of roselle (*Hibiscus sabdariffa* L.) cultivated in Indonesia, namely red and purple roselles [8].

Genetic differences in plant varieties affect their phytochemical content, thus they are qualitatively and quantitatively different. Differences in phytochemical content in plants affect differences in pharmacological activity [9]. Phytochemical content is also influenced by the environment in which plants grow, including geographical location, air conditions, and soil conditions [10]. Figure 1 shows the visual appearance of red and purple roselles.

Figure 1. Appearance of red roselle calyces (left) and purple roselle calyces (right)

The phytochemicals contained in roselle calyces include alkaloids, flavonoids, saponins, tannins, steroids, and triterpenoids [11]. The pharmacological activities of roselle calyces include antidiabetic, antihypertensive, and anticancer [12]. Research has not previously been conducted the on differences in phytochemical content and pharmacological activity in the calyces of different roselle varieties. In this study, the researchers aim to analyze the differences in flavonoid content and antioxidant activity in the calyces of two roselle varieties (*Hibiscus sabdariffa* L.), namely red and purple roselles.

2. Material and Methods

Procedures include extract preparation, total flavonoid analysis, and antioxidant activity testing with slight modification from Yamin et al., 2021. Extract preparation was modified in solvent volume, extraction time, and solvent type. Total flavonoid analysis and antioxidant activity testing were modified in incubation time, reagent concentration, and reagent volume [13].

2.1. Materials

Methanol (Merck), Ethanol (Merck), Quercetin (Merck), Ascorbic Acid (Merck), Aluminium Chloride (Merck), Sodium Acetate (Merck), Demineralized Water (Merck), 2,2-Diphenyl-1-Picrylhydrazyl (Merck), Red and Purple Roselle Calyces (*Hibiscus sabdariffa* L.) obtained from Kolam Village, Percut

Sei Tuan District, Deli Serdang Regency, North Sumatra Province, Postal Code 20371, Republic of Indonesia.

2.2. Tools

Ultraviolet Visible Spectrophotometer (Shimadzu), Ultraviolet Solution Software (Shimadzu), Balance (Tanita), Analytical Balance (Sartorius), Rotary Evaporator (Buchi), Drying Cabinet (Alumex), Cutter (Deli), Filter Paper (Whatman), Vessel (Lion), Parchment Paper (Kirkland), Tissue Paper (Nice), Lens Tissue Paper (Kimtech), Glassware (Iwaki), Microsoft Office (Microsoft).

2.3. Extract preparation

Both red and purple roselle calyces (*Hibiscus sabdariffa* L.) were harvested at 7 to 10 days, washed thoroughly with running water, dried on parchment paper, further dried in a drying cabinet at 50 °C to 60 °C, powdered with a blender, and stored in a tightly closed plastic container. The ethanolic extract of roselle calyces was prepared by maceration. The dried and powdered red and purple roselle calyces were weighed to 500 g and inserted in a vessel to which 5 L 70% ethanol was added. The mixture was stirred frequently for 6 h, soaked for 18 h and filtered. The filtrate was accommodated and the residue inserted again into a vessel, to which 3 L 70% ethanol was added. The mixture was stirred frequently for 6 h, soaked for 18 h, and filtered, after which the filtrate was accommodated. The first filtrate and the second filtrate were combined as dilute extracts, then concentrated with a rotary evaporator at 40 to 50 °C.

2.4. Total flavonoid analysis

The quercetin powder was weighed at 10.0 mg, then dissolved quantitatively in a 100 mL volumetric flask with methanol. Stock solution was obtained at a concentration of 100 µg/mL. The 3.0 mL of the 100 µg/mL quercetin stock solution was diluted quantitatively in a 10 mL volumetric flask (with methanol). A 30 µg/mL solution was obtained, of which 1.0 mL was diluted in a 10 mL volumetric flask (with 3.0 mL methanol, 0.2 mL aluminium chloride 10% solution, 0.2 mL sodium acetate 1 M, and water), and allowed to stand for15 minutes. The absorbance was measured at a wavelength of 400 to 800 nm to obtain a wavelength of maximum absorption (six replications).

A series of 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the 100 µg/mL quercetin stock solution were diluted quantitatively in 10 mL volumetric flasks (with methanol) to obtain a series solution of 10, 20 30, 40, and 50 µg/mL. From the quercetin series solution, 1.0 mL was diluted in a 10 mL volumetric flask (with 3.0 mL methanol, 0.2 mL aluminium chloride 10% solution, 0.2 mL sodium acetate 1 M, and water) and allowed to stand for 15 minutes. The absorbance was measured at a wavelength of maximum absorption. From the obtained data, the regression equation, coefficient of determination, and coefficient of correlation were calculated (six replications).

Each roselle calyces extract (red and purple) was weighed at 50.0 mg and dissolved quantitatively in a 10 mL volumetric flask (with methanol) to obtain the test solution. From each roselle calyces extract test solution, 1.0 mL was diluted in a 10 mL volumetric flask (with 3.0 mL methanol, 0.2 mL aluminium chloride 10% solution, 0.2 mL sodium acetate 1 M, and water) and allowed to stand for 15 minutes. The absorbance was measured at a wavelength that provides maximum absorption. From the obtained data, the total flavonoid content was calculated (six replications).

2.5. Antioxidant activity test

The 2,2-diphenyl-1-picrylhydrazyl powder was weighed to 20.0 mg and dissolved quantitatively in a 100 mL volumetric flask (with methanol) to obtain a stock solution of 200 µg/mL. From the 200 µg/mL 2,2-diphenyl-1-picrylhydrazyl stock solution, 5.0 mL was diluted quantitatively in a 25 mL volumetric flask (with methanol) to obtain a 40 µg/mL concentration. The absorbance was measured at a wavelength of 400 to 800 nm to obtain a wavelength of maximum absorption and operation time (six replications).

The ascorbic acid powder was weighed to 12.5 mg and dissolved quantitatively in a 500 mL volumetric flask (with methanol) to obtain a 25 µg/mL stock solution. From the 25 µg/mL ascorbic acid stock solution, 0.0 mL, 1.5 mL, 3.0 mL, 4.5 mL, 6.0 mL, and 7.5 mL was diluted quantitatively in a 25 mL volumetric flask (with 5 mL of 200 µg/mL 2,2-diphenyl-1-picrylhydrazyl solution and methanol). An ascorbic acid series solution was obtained at 0.0, 1.5, 3.0, 4.5, 6.0, and 7.5 µg/mL with 40 µg/mL 2,2- diphenyl-1-picrylhydrazyl and allowed to stand until operation time. Absorbance was measured at a wavelength of maximum absorption. From the obtained data, free radical scavenging activity and half maximal inhibitory concentration were calculated (by regression equation, coefficient of determination and coefficient of correlation) (six replications).

Each roselle calyces extract (red and purple) was weighed to 125 mg and dissolved quantitatively in a 500 mL volumetric flask (with methanol) to obtain 250 µg/mL stock solution concentration. From each 1000 µg/mL roselle calyces extract, 0.0, 1.5, 3.0, 4.5, 6.0, and 7.5 mL was diluted quantitatively in a 25 mL volumetric flask (with 5 mL of 200 µg/mL 2,2-diphenyl-1-picrylhydrazyl stock solution and methanol). A roselle (red and purple) calyces ethanolic extract series solution was obtained with the concentrations 0.0, 15, 30 , 45, 60, and 75 µg/mL with 40 µg/mL of 2,2-diphenyl-1-picrylhydrazyl, standing until operation time. Absorbance was measured at a wavelength of maximum absorption. From the obtained data, the free radical scavenging activity and half maximal inhibitory concentration were calculated (by regression equation, coefficient of determination and coefficient of correlation) (six replication).

3. Result and Discussion

From the 500 g extraction of dried and powdered red roselle calyces, 83.19 g concentrated extract was obtained, with a reddish brown color and specific odor. From the 500 g extraction of dried and powdered purple roselle calyces, 98.33 g concentrated extract was obtained with a purplish brown color and specific odor.

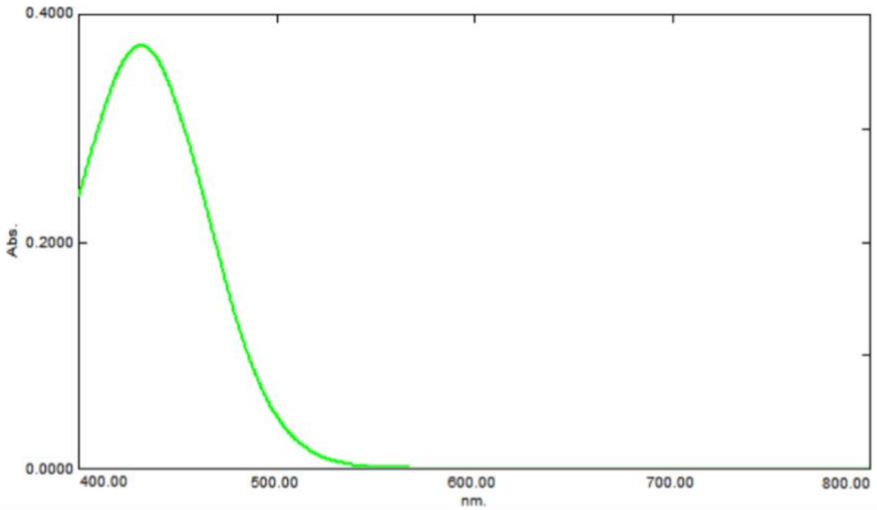
3.1. Total flavonoid analysis

To determine the wavelength of maximum absorption for quercetin measurements, the quercetin absorption curve was measured using quercetin after reaction with aluminum chloride and sodium acetate. The absorption curve of quercetin after reaction with aluminum chloride and sodium acetate, in the wavelength range of 400 to 800 nm, can be seen in Figure 2. The results show that the maximum absorption is at a wavelength of 431.50 nm with an absorption of 0.3839. The wavelength obtained from quercetin is in accordance with the wavelength of quercetin after reaction with aluminum chlor ide and sodium acetate, with an optimal operation time of 15 minutes [14].

To determine the total flavonoid in the sample, which is equivalent to quercetin, the regression equation must be calculated using the quercetin series of 10 to 50 g/mL. From the absorbance obtained from quercetin series the correlation coefficient was also calculated to determine the significance of the relationship between the concentration and the absorbance. The calibration curve of the 10 to 50 g/mL quercetin series can be seen in Figure 3.

The regression equation obtained is as follows: $Y = 0.012858\ X - 0.001160$ with the coefficient of determination (R^2) was 0.999989 and the coefficient of correlation (R) was 0.999995. The coefficient of determination and coefficient of correlation obtained from the quercetin series meets requirements: not less than 0.99, indicating significant relationship between the concentration and the absorbance. These results also indicate that absorbance can be used to determine concentration. That is, absorbance correlates with concentration [15].

Total flavonoid analysis for red and purple roselle calyces ethanolic extract determined 27.55 and 39.18 mg quercetin equivalent per gram. Total flavonoid analysis of red and purple roselle calyces ethanolic extract showed differences in total flavonoids among the two varieties of roselle calyces. Different plant varieties contain phytochemicals that are qualitatively and quantitatively different [16].



No.	P/V	Wavelength (nm)	Absorbance (AU)
1		431.50	0.3839

Figure 2. Absorption curve of quercetin after reaction with aluminum chloride and sodium acetate (400 to 800 nm)

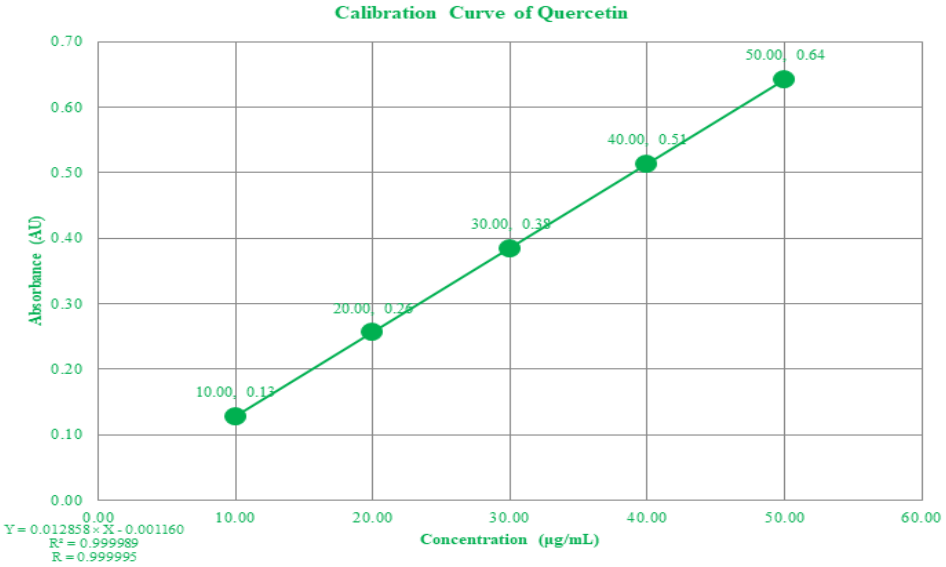
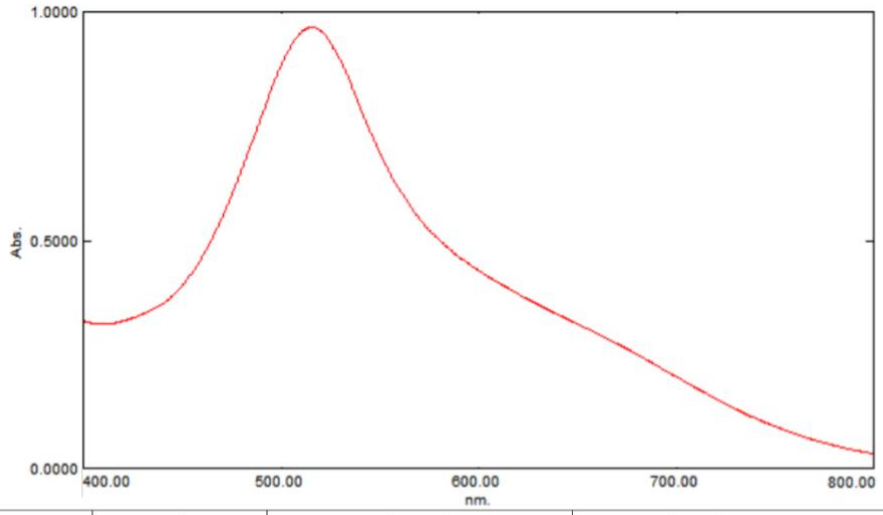


Figure 3. Calibration curve of quercetin series in a concentration range of 10 g/mL to 50 g/mL

3.2. Antioxidant activity test

To determine antioxidant activity, the maximum absorption of 40 µg/mL 2,2-diphenyl-1-picrylhydrazyl solution was measured in methanol solvent using an ultraviolet visible spectrophotometer at 400 to 800 nm. The absorption curve of 2,2-diphenyl-1-picrylhydrazyl at 400 to 800 nm can be seen in Figure 4. The results show that the wavelength of maximum absorption for 2,2-diphenyl-1-picrylhydrazyl was 516.50 nm. The wavelength obtained from 2,2-diphenyl-1-picrylhydrazyl is in accordance with the wavelength of 2,2-diphenyl-1-picrylhydrazyl with an optimal operation time of 30 minutes [17].



No.	P/V	Wavelength (nm)	Absorbance (AU)
1		516.50	0.9813

Figure 4. Absorption curve of 2,2-diphenyl-1-picrylhydrazyl (400 to 800 nm)

Antioxidant activity was further measured by determining the absorption of 40 g/mL 2,2-diphenyl-1-picrylhydrazyl following treatment by ascorbic acid series concentration (1.5 to 7.5 g/mL) and roselle (red and purple roselle) calyces ethanolic extract series concentration (15 to 75 g/mL). The plot of free radical scavenging activity versus concentration from ascorbic acid treatment can be seen in Figure 5. The plot of free radical scavenging activity versus concentration from red roselle calyces ethanolic extract treatment can be seen in Figure 6. The plot of free radical scavenging activity versus concentration from purple roselle calyces ethanolic extract treatment can be seen in Figure 7

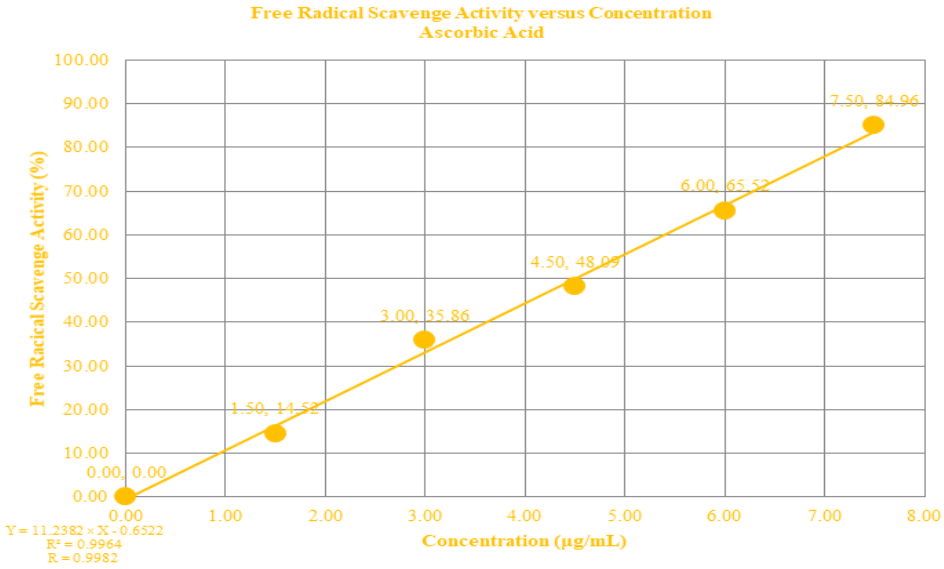


Figure 5. Free radical scavenging activity versus concentration from ascorbic acid treatment

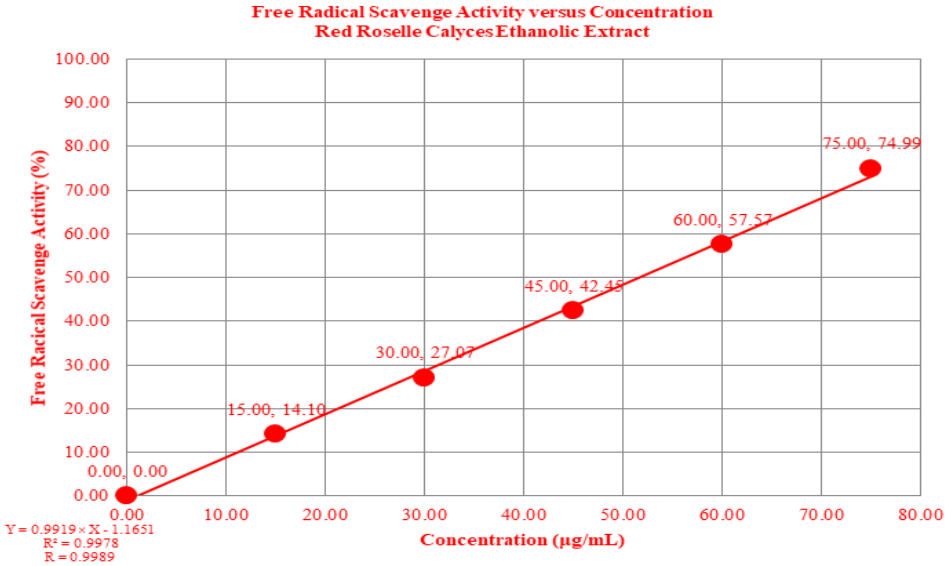


Figure 6. Free radical scavenging activity versus concentration from red roselle calyces ethanolic extract treatment

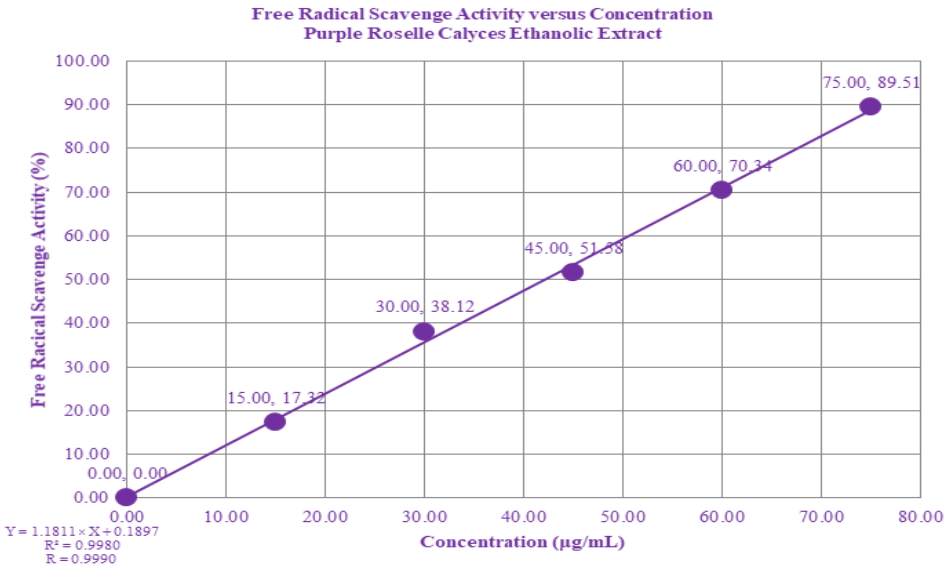


Figure 7. Free radical scavenging activity versus concentration from purple roselle calyces ethanolic extract treatment

Absorbance measurement from the series concentration of ascorbic acid, red roselle calyces ethanolic extract, and purple roselle calyces ethanolic extract produced regression equations as follows: Y = 11.2382X - 0.6522; Y = 0.9919X - 1.1651; and Y = 1.1811X + 0.1897. The coefficients of determination (R^2) were 0.9964, 0.9978, and 0.9980. The coefficients of correlation (R) were 0.9982, 0.9989 and 0.9990. All the coefficients of determination and coefficient of correlations were not less than 0.99, meeting the requirement. Thus, the concentration can be used to determine free radical scavenging activity as concentration correlates with free radical scavenging activity. These results also mean that free radical scavenging activity is concentration dependent [18].

Absorbance value decreases because the test solution (ascorbic acid or roselle calyces ethanolic extract) traps the 2,2-diphenyl-1-picrylhydrazyl which acts as a free radical. Scavenging activity occurs

due to compounds that function as radical scavengers to reduce and neutralize 2,2-diphenyl-1-picrylhydrazyl. This reaction is observed by a change in color intensity. When odd electrons pair with hydrogen from free radical scavenging compounds, the purple or yellow color intensity decreases [19].

Half maximal inhibitory concentration of ascorbic acid, red roselle calyces ethanolic extract, and purple roselle calyces ethanolic extract were further calculated by regression equation, obtained from absorbance measurement from series concentration. The half maximal inhibitory concentration values for ascorbic acid, red roselle calyces ethanolic extract, and purple roselle calyces ethanolic extract were 4.51, 51.59, and 42.17 µg/mL.

The half maximal inhibitory concentration value is inversely proportional to antioxidant activity. The lower the half maximal inhibitory concentration value, the stronger the free radical scavenging activity, and the stronger the antioxidant activity. Ascorbic acid has stronger antioxidant activity than red roselle calyces ethanolic extract and purple roselle calyces ethanolic extract. Ascorbic acid antioxidant activity is stronger because ascorbic acid is a pure compound, while the extract remains a mixture of several compounds of varying amounts [20].

The results showed that both ascorbic acid and roselle calyces ethanolic extract have antioxidant activity. A substance has antioxidant properties if the half maximal inhibitory concentration value is less than 500 µg/mL [21]. The results showed that both purple and red roselle calyces ethanolic extract had very strong antioxidant activity. The strength of antioxidant activity can also be determined based on the half maximal inhibitory concentration value, declared "very strong" if less than 50 µg/mL, "strong" from 50 to 100 µg/mL, "medium" from 100 to 500 µg/mL, and "weak" if more than 150 µg/mL [22].

The antioxidant activity of purple roselle calyces ethanolic extract was better than red roselle calyces ethanolic extract. This may be due to phytochemical content that is qualitatively and quantitatively different. Differences in plant varieties cause qualitative and quantitative differences in phytochemical content, causing differences in pharmacological activity in vitro and in vivo [23]. Antioxidants play a role in preventing tissue damage caused by free radicals by eliminating the formation of radicals or reducing free radicals [24]. Antioxidant activity in roselle calyces ethanolic resulted from several phytochemical secondary metabolites, namely flavonoids, active compounds in antioxidant activity. Demonstrating antioxidant activity, roselle calyces ethanolic extract can be used as a source of antioxidants needed by the body [25].

4. Conclusion

Different varieties in roselle calyces ethanolic extract caused differences in total flavonoid content and antioxidant activity. The total flavonoid content and antioxidant activity of purple roselle calyces ethanolic extract was higher and stronger than red roselle calyces ethanolic extract.

Acknowledgement

The authors express appreciation to the Institut Kesehatan Deli Husada Deli Tua for research grant support and Mutiara Mukti Farma for material support.

Conflict of Interest

The authors declare there was no conflict of interest in this research and no conflict of interest in this manuscript.

References

[1] Phaniendra A, Jestadi D B, and Periyasamy L 2015 *Indian J Clin Biochem* **30** 11-26
[2] Sharifi-Rad M, Kumar N V A, Zucca P, Varoni E M, Dini L, Panzarini E, Rajkovic J, Fokou P V T, Azzini E, Peluso I, Mishra A P, Nigam M, Rayess Y E, Beyrouthy M E, Polito L, Iriti M, Martins N, Martorell M, Docea A O, Setzer W N, Calina D, Cho W C, and Sharifi-Rad J 2020 *Front Physiol* **11** 694
[3] Parcheta M, Swisłocka R, Orzechowska S, Akimowicz M, Choinska R, and Lewandowski W

2021 *Materials* **14** 1984
[4] Manessis G, Kalogianni AI, Lazou T, Moschovas M, Bossis I, and Gelasakis A I 2020 *Antioxidants* **9** 1215
[5] Kopustinskiene D M, Jakstas V, Savickas A, and Bernatoniene J 2020 *Nutrients* **12** 457
[6] Panche A N, Diwan A D, and Chandra S R 2016 *J Nutr Sci* **5** e47
[7] Riaz G, and Chopra R 2018 *Biomed Pharmacother* **102** 575-586
[8] Hapsari B W, Manikharda M, and Setyaningsih W 2021 *Horticulturae* **7** 35
[9] Campbell O E, Merwin I A, and Padilla-Zakour O I 2013 *J Agric Food Chem* **61** 12700-12710
[10] Zargoosh Z, Ghavam M, Bacchetta G, and Tavili A 2019 *Sci Rep* **9** 16021
[11] Adusei S 2020 *IPTEK J Tech Sci* **31** 139-47
[12] Izquierdo-Vega J A, Arteaga-Badillo D A, Sánchez-Gutiérrez M, Morales-González J A, Vargas-Mendoza N, Gómez-Aldapa C A, Castro-Rosas J, Delgado-Olivares L, Madrigal-Bujaidar E, and Madrigal-Santillán E 2020 *Biomedicines* **8** 100
[13] Yamin Y, Ruslin R, Mistriyani M, Sabarudin S, Ihsan S, Armadany F I, Sahumena M H, and Fatimah W O N 2021 *Food Res* **5** 84-90
[14] Affes S, Younes A B, Frikha D, Allouche N, Treilhou M, Tene N, and Mezghani-Jarraya R 2021 *Molecules* **26** 4338
[15] Ekasari W, Widiyastuti Y, Subositi D, Hamsidi R, Widyawaruyanti A, Basuki S, and Setyawan D 2020 *Sci World J* **2020** 7367836
[16] Wang M, Liu X, Zhang Z, Yu J, Liu J, and Wu Y 2020 *J Food Biochem* **45** e13183
[17] Sinala S, Ibrahim I, Salasa A M 2020 *Pharmacogn J* **12** 1340-5
[18] Morabbi Najafabad A, and Jamei R 2014 *Avicenna J Phytomed* **4** 343-53
[19] Flieger J, and Flieger M 2020 *Molecules* **25** 6005
[20] Matuszewska A, Jaszek M, Stefaniuk D, Ciszewski T, Matuszewski Ł 2018 *Cerrena unicolor Plos One* **13** e0197044
[21] Santos C M M, and Silva A M S 2020 *Molecules* **25** 696
[22] Marjoni M R, and Zulfisa A 2017 *Pharm Anal Acta* **8**1 000557
[23] Altemimi A, Lakhssassi N, Baharlouei A, Watson D G, and Lightfoot D A 2017 *Plants* **6** 42
[24] Kurutas E B 2015 *Nutr J* **15** 71
[25] Jabeur I, Pereira E, Barros L, Calhelha R C, Soković M, Oliveira M B P P, Ferreira I C F R 2017 *Food Res Int* **100** 717-23