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QUANTIFICATION OF TOTAL FLAVONOID CONTENT IN FRACTIONATED YOUNG LEAF EXTRACTS OF RED SHOOT LEAVES (*Syzygium myrtifolium*) USING UV-VIS SPECTROPHOTOMETRY

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ABSTRACT

Syzygium myrtifolium (red shoot leaves) is widely known for its rich phytochemical profile, particularly flavonoids, which exhibit various pharmacological properties. This study aimed to determine the total flavonoid content in the crude extract, n-hexane fraction, ethyl acetate fraction, and water fraction of red shoot leaves (*Syzygium myrtifolium*) using a colorimetric method with quercetin as a standard. The results revealed that the ethyl acetate fraction had the highest flavonoid content (65.781 ± 6.365 mgQE/g), followed by the crude extract (26.093 ± 0.961 mgQE/g), n-hexane fraction (18.293 ± 2.925 mgQE/g), and water fraction (12.583 ± 0.824 mgQE/g). The study also found that the solvent polarity significantly influenced the flavonoid extraction efficiency, with ethyl acetate, a moderately polar solvent, being most effective for isolating flavonoid aglycones. The n-hexane fraction contained polymethyl flavonoids, which are more soluble in nonpolar solvents, while flavonoid glycosides were more soluble in polar solvents like water and alcohol mixtures. Furthermore, the research highlights the importance of extraction methods, as the total flavonoid content in mature red shoot leaves was found to be higher than in younger leaves, likely due to increased secondary metabolite production in mature tissues. This study emphasizes the role of solvent polarity in flavonoid extraction and provides insights into the phytochemical composition of *S. myrtifolium* leaves for potential applications in natural product research.

Keywords: ethanol, fractionation, red shoot leaves, total flavonoid, young leaf

INTRODUCTION

Syzygium myrtifolium Walp., commonly known in Indonesia as the red shoot plant, belongs to the Myrtaceae family. The tropical climate of Indonesia provides ideal conditions for its growth. Characterized by its vibrant red leaf shoots, this plant is widely cultivated as an ornamental species (Sunarti, 2021). It is commonly found along roadsides, in home gardens, office complexes, and city parks (Anggraini, 2017).

Red shoots are rich in secondary metabolites, which exhibit various pharmacological properties, including antioxidant, antibacterial, antifungal, and antiviral activities (Ahmad et al., 2022). The plant is particularly abundant in flavonoids, such as dimethyl cardamonin, anthocyanin, and luteolin, which have shown potential as anticancer agents and antioxidants (Santoni et al., 2013; Memon et al., 2014; Kusriani et al., 2019).

Flavonoids are a class of polyphenolic compounds commonly found in plants, where they contribute to the pigmentation of leaves, flowers, and fruits (Lubis et al., 2020; Nurlinda et al., 2021). These compounds are water-soluble and exhibit color changes when exposed to bases or ammonia. Structurally, flavonoids are derivatives of the parent compound flavone and share similar chemical properties with other derivatives. The presence of conjugated aromatic systems in flavonoids is responsible for their strong absorption bands in the UV-Vis spectrum (Harborne, 1987).

Fractionation involves separating an extract into multiple fractions, each containing specific secondary metabolite compounds. This process can be carried out to the point of obtaining pure compounds, which are subsequently isolated and identified (Abubakar & Haque, 2020). Fractionation plays a crucial role in grouping analytes from a sample based on their physical or chemical properties. The choice of solvent used in this process significantly influences the types of secondary metabolites that are extracted (Nugroho, 2017).

Several studies have investigated the total flavonoid content in red shoot (*Syzygium myrtifolium* Walp.) leaves. Ahmad et al. (2022) reported that the ethanol and water extracts of mature red shoot leaves contained total flavonoid levels of 14.2% and 25.0% (v/v) QE, respectively. In contrast, Memon et al. (2014) observed higher flavonoid levels in similar extracts, with the ethanol extract containing 68.8% (m/m) QE and the water extract containing 44.9% (m/m) QE. These variations suggest potential differences in extraction methods, environmental factors, or plant maturity that may influence flavonoid content.

Based on the above description, research on the total flavonoid content of red shoot (*Syzygium myrtifolium* Walp.) leaves has been primarily focused on extracts from mature leaves. This has prompted researchers to expand their studies to evaluate the total flavonoid content in the extract, as well as in the n-hexane, ethyl acetate, and water fractions of young red shoot (*Syzygium myrtifolium* Walp.) leaves.

RESEARCH METHOD

Equipments and Materials

The equipment used in this research included a blender (Philips), mesh sieve No. 60 (CV Putra Masagus), microscope (Leica), analytical balance (Kenko), filter paper (Sartorius), vacuum rotary evaporator (Eyela), water bath (Memmert), micropipette (Watson Bio Lab), cuvette (Hellma), and UV-Vis spectrophotometer (Shimadzu UV-1800).

The test material consisted of two-week-old young leaves of red shoot (*Syzygium myrtifolium*), sourced from the Bogor Spice and Medicinal Plant Research Institute (BALITTRO). The plant material was formally identified at the Department of Biology, Universitas Indonesia, Depok, under letter number 955/UN2.F3.11/PDP.02.00/2022.

The chemicals used in the research were distilled water, n-hexane, ethyl acetate, 96% ethanol, H_2SO_4, 2 N HCl, Dragendorf's reagent, Mayer's reagent, Wagner's reagent, 5% $NaNO_2$, 10% NaOH, HCl, chloroform, ether, anhydrous acetic acid, 1% $FeCl_3$, 10% $AlCl_3$, quercetin (Sigma Aldrich), ethanol p.a. (Mallinckrodt), and 1 M CH_3COOK.

Research Path

1. Sample Processing

Three kilograms of fresh leaves were washed with running water until clean and then air-dried for one week. The dried samples were sorted, ground into powder using a blender, and then sieved through mesh No. 60. The resulting powder was stored in a tightly closed container and labeled.

2. Extraction and Fractionation Process of Young Red Shoot Leaves.

A total of 100 g of sample powder was weighed and macerated with 96% ethanol at a 1:10 ratio for 24 hours, with occasional stirring. The stirring was done for the first 6 hours, and the mixture was left to stand for the remaining 18 hours.

The maceration process was repeated twice using the same solvent, but with half the volume of the initial maceration. The resulting extracts were filtered and then evaporated using a vacuum rotary evaporator at 45°C for 4 days. Afterward, the extracts were concentrated using a water bath until a constant mass was achieved. The final extract was weighed, and the yield was calculated based on the weight of the initial simplicia powder (Kemenkes RI, 2017).

The fractionation process began by weighing 40 g of the thick solvent-free extract, which was then dissolved in 500 mL of water and partitioned with 500 mL of n-hexane using a separating funnel. The funnel was shaken vigorously, occasionally

opening the lid to release any gas formed. The separating funnel was placed on a stand to remain stable until two distinct layers were formed. This partitioning was repeated three times, resulting in the n-hexane fraction and the water fraction. Next, the water fraction was partitioned again using 500 mL of ethyl acetate. The ethyl acetate fractionation process was carried out in the same manner as the n-hexane partitioning. The ethyl acetate fraction was collected in a separate container. This process yielded three extract fractions: the n-hexane fraction, the ethyl acetate fraction, and the water fraction. Finally, all three fractions were evaporated to remove the solvent, obtaining thick solvent-free fractions (Sukmawati et al., 2018).

3. Phytochemical Screening

Phytochemical screening tests were performed on the powder, extract, and fractions (n-hexane, ethyl acetate, and water) of young red shoot leaves. These tests included the identification of alkaloids, flavonoids, saponins, tannins, and triterpenoids/steroids (Depkes RI, 1995; Syafriana & Wiranti, 2022).

4. Total Flavonoid Content

A total of 20 mg of extract, n-hexane fraction, and water fraction were each dissolved in 10 mL of ethanol p.a., while 10 mg of the ethyl acetate fraction was dissolved in 10 mL of ethanol p.a. The difference in sample weights, particularly for the ethyl acetate fraction, was adjusted based on absorbance values. At a concentration of 20 mg/mL, errors in absorbance readings may have occurred due to the high flavonoid content in the ethyl acetate fraction. Next, 1 mL of each sample was pipetted from the stock solution into a 10 mL volumetric flask, followed by the addition of 3 mL of ethanol p.a., 0.2 mL of 10% $AlCl_3$, 0.2 mL of CH_3COOK, and distilled water up to the mark.

The sample solution was incubated, and its absorbance was measured using a UV-Vis spectrophotometer at the maximum wavelength of quercetin. Three replicate samples were analyzed for each test, and the average absorbance value was calculated (Chang et al., 2002).

The data obtained were then analyzed quantitatively using a UV-Vis spectrophotometer to determine the total flavonoid content in the extracts and fractions of young red shoot leaves. A concentration series of quercetin standards was used to create a standard curve equation for total flavonoids. The standard curve equation was $y = bx + a$, where y represents absorbance (in nm) and x represents flavonoid concentration. The absorbance values of the ethanol extract and the

fractions of young red shoot leaves were then applied to the standard curve equation to calculate the total flavonoid content.

RESULTS AND DISCUSSION

1. Samples Processing

The sample processing includes wet sorting, drying, dry sorting, and grinding. Proper processing of medicinal plant samples is crucial for producing simplicia that meets quality standards, ensuring the preservation of active ingredient content (Susanti & Safrina, 2021).

Wet sorting is performed immediately after fresh material is obtained, prior to washing. This step aims to separate the plant material from impurities such as soil, weeds, rotten or damaged plant parts, and unused portions. The goal of wet sorting is to maintain the purity of the material, minimize initial contamination that could affect subsequent processes, and reduce microbial contamination (Susanti & Safrina, 2021; Panaungi & Sakka, 2022; Pangondian et al., 2023).

The next stage is washing, which removes dirt and contaminants adhered to the leaves. This process is carried out using running water to ensure that contaminants are effectively removed and do not reattach, ensuring the leaves are thoroughly clean and suitable for research (Susanti & Safrina, 2021; Panaungi & Sakka, 2022; Pangondian et al., 2023).

The washed samples are then drained and air-dried. Air-drying was chosen for this research to prevent the loss of thermolabile compounds, such as flavonoids, during the drying process (Syafriana et al., 2021). Since the young red shoot leaves contain anthocyanins, which are part of the flavonoid group, it is crucial to maintain this drying method to minimize the loss of active compounds from the leaves (Priamsari et al., 2016; Syafriana & Wiranti, 2022).

The dried samples are then ground into a powder and sieved to increase the surface area, aiding in the breakdown of cell walls and membranes for more efficient extraction. Additionally, sieving with a consistent mesh size ensures a uniform particle size, allowing for even and optimal solvent diffusion into the sample (Novi et al., 2023).

2. Process of Extraction and Fractionation of Young Red Shoot Leaves

The thick free solvent extract obtained weighed 55.17 g, resulting in a yield of 55.17%. This relatively high yield indicates the substantial number of metabolite compounds present in the extract (Kusuma et al., 2022). However, this yield is lower than that reported in previous research,

which showed an 81% yield for young red shoot leaf extract using 70% ethanol as the solvent (Syafriana & Wiranti, 2022). This difference may be due to the higher polarity of 70% ethanol, which can extract a broader range of compounds compared to 96% ethanol. Generally, the higher the solvent's polarity, the greater the yield (Azzahra & Budiati, 2022). Nonetheless, the yield of over 50% still reflects the considerable amount of active compounds in the young red shoot leaf extract.

Fractionation was performed using solvents with varying polarities to separate compounds based on their affinity for each solvent. This process began with a low-polarity (nonpolar) solvent to gradually bind the compounds, preventing the immediate attraction of all compounds by the more polar solvent. The ability of different solvents to bind compounds with distinct polarities means that fractionation using polar solvents is typically conducted last (Wijaya et al., 2023). The yields of the n-hexane, ethyl acetate, and water fractions were 14.34%, 30.73%, and 21.02%, respectively (Table 1). These results indicate that the ethyl acetate fraction had the highest yield, followed by the water fraction, while the n-hexane fraction yielded the least.

The high yield observed in the ethyl acetate fraction aligns with the findings of Febriani et al. (2022) and Kusuma et al. (2022). These results suggest that the young red leaves of the red shoot plant contain a higher concentration of semi-polar compounds compared to polar and non-polar compounds. Ethyl acetate is particularly effective for extracting semi-polar secondary metabolites, such as flavonoids and tannins (Tanaya et al., 2015). This is consistent with the understanding that flavonoids are the dominant secondary metabolites in red shoots, particularly in the young leaves.

3. Phytochemical Screening

The results of the phytochemical screening are presented in Table 1.

Table 1. Phytochemical screening results of powder, ethanol extract, and fractions from young red shoot leaves

Compounds Identification	Simplisia (powder)	Extract	*n*-hexane Fraction	Ethyl Acetate Fraction	Water Fraction
Alkaloid	-	-	-	-	-
Flavonoid	+	+	+	+	+
Saponin	+	+	-	-	+
Tannin	+	+	+	+	+
Triterpenoid	+	+	+	+	+
Steroid	-	-	+	-	-

Notes:
(+): The identified compounds were detected in the samples
(-): The identified compounds were not detected in the samples

Phytochemical screening was conducted to determine the presence of secondary metabolite compounds in the powder, crude extract, and young leaf fractions of red shoots (*Syzygium myrtifolium* Walp.). As shown in Table 1, all samples tested negative for alkaloids. The alkaloid test typically results in the formation of a potassium-alkaloid complex precipitate if alkaloids are present. This precipitate forms through a precipitation reaction, where alkaloid compounds form covalent bonds with K^+ ions via coordination with the nitrogen atom (Fadiyah et al., 2019).

All samples tested positive for flavonoid identification. The addition of $NaNO_2$ serves as a nitrating agent, producing flavonoid-nitroxyl derivatives that exhibit an orange color. The subsequent addition of $AlCl_3$ facilitates the formation of a flavonoid-Al(III) complex, and the addition of NaOH induces a color change in the solution to pink-red (Shraim et al., 2021).

The powders, crude extract, and water fraction samples all showed positive results for saponin identification. Saponins possess a hydrophilic group that bonds with water, while their hydrophobic group repels water or bonds with air. This characteristic leads to the formation of micelles when the samples are shaken, resulting in hydrolysis (Kumaradewi et al., 2021). The foam formation indicates the presence of glycosides, which, upon hydrolysis, produce glucose and aglycones (Parbuntari et al., 2018). The addition of 2 N HCl increases polarity, enhancing the stability of the hydrophilic group's bonding and stabilizing the foam (Tandi et al., 2020).

All five samples showed positive results for tannin identification. The phenolic compounds in tannins react with Fe^{3+} ions to form complex compounds,

which is responsible for the color change observed during the test (Ningsih, 2017).

All five samples also showed positive results for triterpenoid identification. However, only the n-hexane fraction tested positive for steroid identification. The reaction between steroids and anhydrous acetic acid involves acetylation of the –OH group on the steroid, forming an acetyl steroid complex. The addition of H_2SO_4 facilitates the hydrolysis of water, which reacts with the acetyl derivatives to produce a colored solution. This color change is indicative of the presence of steroids when combined with anhydrous acetic acid and H_2SO_4 (Kumaradewi et al., 2021).

According to research conducted by Haryati et al. (2015), several differences were observed in the results of phytochemical screening, such as the absence of alkaloid content in the extract, n-hexane fraction, and ethyl acetate fraction. Additionally, flavonoids were detected in the n-hexane fraction, a finding that contrasts with the results of Haryati et al. (2015), where a different flavonoid test method was employed.

4. Total Flavonoid Content

4.1 Determination of Quercetin as a Standard for Operational Time

In this study, flavonoid levels were determined using quercetin as the standard. Quercetin, a flavonol widely distributed in various plants (Nurlinda et al., 2021), along with its glycosides, constitutes a significant portion of flavonoids, comprising approximately 60-75%. Quercetin's ability to form complex compounds by binding with $AlCl_3$ is key to its role in flavonoid quantification (Winahyu et al., 2019).

Before measuring the absorbance of the sample, the optimal operating time for quercetin was determined to ensure that the reference and sample solutions were incubated for the correct duration until the absorbance became stable. The quercetin solution was monitored for 45 minutes, and stable absorbance values were observed between the 26^{th} and 33^{rd} minute. These results are consistent with the findings of Suharyanto & Hayati (2021), who reported stable absorbance values from the 28^{th} minute to the 32^{nd} minute.

4.2 Determination of the Maximum Absorption Wavelength of Quercetin

In addition to determining the optimal operating time, the maximum wavelength (λ_{max}) of quercetin was also established. The wavelength was measured across a range of 400-800 nm, with a peak absorption occurring at 428 nm. The analysis was conducted at this maximum wavelength because it provides the highest sensitivity,

leading to significant changes in absorption and resulting in a linear curve. This ensures minimal experimental error upon repetition and satisfies the Lambert-Beer law (Suharyanto & Hayati, 2021).

4.3 Construction of a Quercetin Standard Calibration Curve

The calibration curve was determined by preparing five different quercetin concentration series: 4, 5, 6, 7, and 8 ppm (Figure 1). The purpose of constructing the calibration curve is to obtain a linear regression equation, which allows for the calculation of flavonoid levels present in the sample.

The quercetin standard curve yielded a linear regression equation of y = 0.05983x + 0.05910, with an r^2 value of 0.98143 and a correlation coefficient (r) of 0.99067, indicating a strong linear relationship between quercetin concentration and absorbance (Figure 1) (Nofita et al., 2020). The correlation coefficient (r), which is close to 1, confirms a strong relationship between the variables, suggesting that the data points align closely along a linear curve with high confidence (Winahyu et al., 2019). The standard curve results further demonstrate that as quercetin concentration increases, absorbance also increases, reinforcing the direct linear relationship (Bangun et al., 2021).

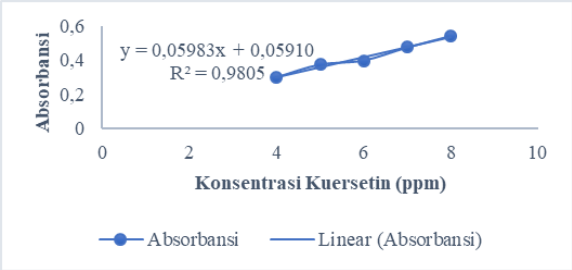

Figure 1. Linear regression plot of the quercetin standard curve

4.4 Quantification of Total Flavonoid Content

Determining the total flavonoid levels aims to quantify the flavonoid compounds present in the extracts and fractions of young red shoot leaves (*Syzygium myrtifolium* Walp.). This was achieved using the colorimetric method, which relies on the formation of a color change. The colorimetric method with $AlCl_3$ is preferred because it forms a complex compound with the keto group on the C-4 atom and the hydroxyl group on the C-3 or C-5 atom of flavonol and flavonoid groups (Fadillah et al., 2017). The addition of $AlCl_3$ is crucial as it binds with quercetin to form a stable complex, particularly with the orthohydroxyl group on the A- or B-ring of the flavonoid compound (Winahyu *et al.*, 2019). Furthermore, $AlCl_3$ induces a shift in the absorption spectrum towards the visible region, causing a noticeable color change to yellow (Lindawati & Ma'ruf, 2020).

The addition of CH_3COOK serves to stabilize the solution and maintain the

wavelength within the visible spectrum (Lindawati & Ma'ruf, 2020). Additionally, CH_3COOK helps to detect the presence of 7-hydroxyl groups in flavonoids (Nofita et al., 2020). To protect the complex compound from light sensitivity, the solution was incubated in a dark environment(Lindawati & Ma'ruf, 2020). Figure 2 illustrates the reaction between quercetin and $AlCl_3$, leading to the formation of the complex compound (Lindawati & Ma'ruf, 2020).

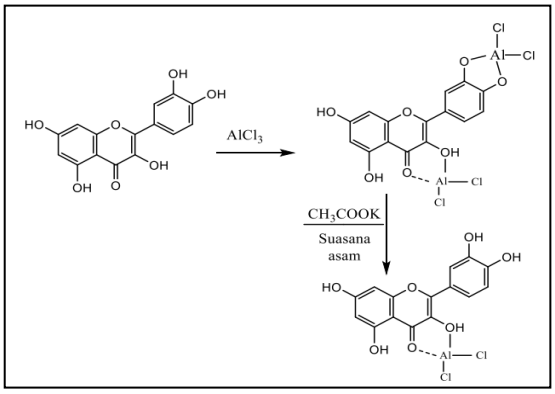

Figure 2. Reaction of quercetin with $AlCl_3$ and CH_3COOK
[Source: Lindawati & Ma'ruf, 2020]

Replication was performed to assess the standard deviation of the absorbance values obtained from the extract, n-hexane fraction, ethyl acetate fraction, and water fraction of young red shoot leaves (*Syzygium myrtifolium* Walp.).

Based on Table 3, the ethyl acetate fraction has the highest total flavonoid content at 65.781 ± 6.365 mgQE/g, followed by the crude extract, which ranks second. The n-hexane fraction and the water fraction have total flavonoid contents of 18.293 ± 2.925 mgQE/g and 12.583 ± 0.824 mgQE/g, respectively.

The polarity of flavonoid compounds is determined by the number and position of hydroxyl groups, which in turn affects their solubility (Harborne, 1987). The solubility of flavonoids varies depending on the polarity of both the compound and the solvent (Dias et al., 2021). In this study, the polarity of the flavonoids in the extract, n-hexane fraction, ethyl acetate fraction, and water fraction closely matches the polarity of the solvents used.

The ethyl acetate fraction exhibited the highest total flavonoid content despite being tested at half the concentration (10 mg/mL) compared to the other extracts and fractions (20 mg/mL each). This is likely due to the presence of flavonoid compounds that are less polar, such as flavonoid aglycones. Flavonoid aglycones include several classes, such as isoflavones, flavanones, flavonols, and methylated flavanones (Andersen & Markham, 2005; Tanaya et al., 2015; Irianti et al., 2021). Methylation in flavonoids occurs when a methyl group bonds with the aglycone via oxygen or carbon atoms, resulting in O-methylated or C-methylated compounds (Koirala et al., 2016). These flavonoid

derivatives are soluble in solvents like chloroform, dichloromethane, diethyl ether, or ethyl acetate (Andersen & Markham, 2005).

Table 3. Total flavonoid content in crude extract, n-hexane fraction, ethyl acetate fraction, and water fraction of young red shoot plant leaves

Sample	Replication	Absorbance (y)	TFC (mgQE/g extract)	Average of TFC (mgQE/g extract)	SD
Crude Extract	1	0.383	27.068	26.093	0.961
	2	0.371	26.066		
	3	0.360	25.146		
n-hexane fraction	1	0.278	18.293	18.293	2.925
	2	0.313	21.218		
	3	0.243	15.369		
Ethyl Acetate Fraction	1	0.419	60.154	65.781	6.365
	2	0.494	72.689		
	3	0.445	64.499		
Water Fraction	1	0.203	12.026	12.583	0.824
	2	0.205	12.193		
	3	0.221	13.530		

In contrast, the extract and water fraction, which used polar solvents, exhibited lower total flavonoid levels than the ethyl acetate fraction. This suggests that only a limited number of flavonoid compounds were able to dissolve in the polar solvents, resulting in a lower total flavonoid content. Flavonoid glycosides, which are more soluble in alcohol, water, or alcohol-water mixtures, likely dissolved more readily in the extract and water fractions of young red shoot leaves (Markham, 1988; Andersen & Markham, 2005). The presence of sugar bound to flavonoids in their structure facilitates their solubility in polar solvents (Markham, 1988).

Furthermore, Doloking et al. (2022) reported that alcohol concentration plays a significant role in flavonoid extraction, with high alcohol concentrations (90–95%) favoring the extraction of free flavonoids, while lower concentrations (around 60%) are more effective for flavonoid glycosides. These findings suggest that the flavonoid compounds present in the crude extract are predominantly free flavonoids, given the solvents and extraction conditions used. This explanation highlights the relationship between solvent polarity and flavonoid composition, providing insights into the distribution of flavonoid types across the different fractions.

The n-hexane fraction had the third highest total flavonoid content, following

the ethyl acetate fraction and crude extract. Nonpolar solvents, such as n-hexane, petroleum ether, chloroform, and ether, are effective at dissolving polymethyl flavonoid compounds (Wahyusi *et al.*, 2020). The presence of methyl groups in flavonoids enhances their hydrophobic properties, and the more methyl groups present, the greater the impact on the flavonoid's polarity. This increased hydrophobicity allows these compounds to dissolve more effectively in nonpolar solvents (Wen *et al.*, 2017).

Based on previous research, the total flavonoid content in the ethanol extract of mature red shoot leaves was reported as 141.6 mgQE/g and 688.8 mgQE/g. This difference in levels is attributed to variations in extraction methods, specifically maceration and soxhlet extraction (Memon *et al.*, 2014; Ahmad *et al.*, 2022). Extraction efficiency can be influenced by temperature, with higher temperatures generally resulting in faster mass transfer and greater extraction yields. However, when compared to the young leaves of red shoots, the total flavonoid content in mature leaves remains higher. This is because, as plants mature, they produce more secondary metabolites, including flavonoids (Najib, 2018).

Thus, the characteristics of the flavonoid compounds in each sample exhibit polar properties that align with the polarity of the solvent used. In this study, the flavonoid compounds in the ethyl acetate fraction had a polarity similar to that of ethyl acetate. Similarly, the extract, n-hexane fraction, and water fraction demonstrated polarities corresponding to those of their respective solvents.

CONCLUSION

In conclusion, the study successfully determined the total flavonoid content in various extracts and fractions of *Syzygium myrtifolium* (red shoot leaves). The ethyl acetate fraction exhibited the highest flavonoid content, likely due to its ability to dissolve less polar flavonoid aglycones. In contrast, the water and n-hexane fractions had lower flavonoid levels, reflecting the solubility characteristics of the respective solvents. The results highlight the influence of solvent polarity on flavonoid extraction efficiency, with ethyl acetate proving to be the most effective for isolating flavonoid compounds in this study.

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