HYDROGEN
JURNAL KEPENDIDIKAN KIMIA
https://e-journal.undikma.ac.id/index.php/hydrogen/index

June 2025. 13(3)
e-ISSN: 2656-3061
p-ISSN: 2338-6487
pp. 416-427

Comparative Analysis of Antioxidant Potential in Hexane and Methanol Fractions of Sungkai Leaves

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Article History
Received: 16-05-2025
Revised: 26-05-2025
Published: 30-6-2025

Keywords: *Paronema canescens* Jack; antioxidant activity; phenolic; RNS; ROS

Abstract
This study aimed to compare the antioxidant potential of hexane and methanol fractions of sungkai (*Paronema canescens* Jack) leaves from Tanah Datar Regency, West Sumatra, Indonesia. The methanol extract was then fractionated using hexane and ethyl acetate. Total phenolic content was determined using the Folin-Ciocalteu method and antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. According to the conducted research, the methanol fraction exhibits a significantly higher total phenolic content, quantified at 92.22 mg GAE/g, in contrast to the hexane fraction, which recorded a content of 25.33 mg GAE/g. Furthermore, the methanol fraction demonstrated robust antioxidant activity, with an IC50 value of 75.425 mg/L, whereas the hexane fraction demonstrated comparatively weaker antioxidant activity, with an IC50 value of 232.595 mg/L. The strong antioxidant activity of the methanol fraction can be attributed to its high content of phenolic compounds, such as flavonoids and tannins, which are known to possess potent antioxidant properties. The synergistic effects of these compounds may further enhance the overall antioxidant capacity of the methanolic fraction. In conclusion, the methanol fraction of sungkai leaves from Tanah Datar Regency demonstrates superior antioxidant potential compared to the hexane fraction, suggesting its potential as a natural source of antioxidants for various applications.

How to Cite: Deswita, E., Imelda, I., & Suryati, S. (2025). Comparative Analysis of Antioxidant Potential in Hexane and Methanol Fractions of Sungkai Leaves. Hydrogen: Jurnal Kependidikan Kimia, 13(3), 416-427. doi:https://doi.org/10.33394/hjkk.v13i3.15692

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INTRODUCTION

Antioxidant activity pertains to the capacity of compounds to neutralize deleterious free radicals and reactive oxygen species (ROS) in biological systems. This attribute is crucial for mitigating oxidative stress-related diseases and promoting overall health (Jung et al., 2023). Numerous plants have been extensively utilized as natural antioxidants, including the leaves of the sungkai (*Paronema canescens* Jack). Sungkai are indigenous to tropical regions with high precipitation such as Indonesia. It is a traditional medicinal plant commonly used in the treatment of hypertension, hypercholesterolemia (Pratiwi et al., 2021), toothache, fever, stomach pain, skin care, and malaria (Muharni et al., 2021).

Based on the literature, there is much scientific information on the chemical content and biological activity of sungkai plants. Phytochemical tests of hexane, ethyl acetate, and methanol extracts of fresh sungkai leaves showed that this plant contains flavonoids, phenolics, saponins, triterpenoids, steroids, and alkaloids (Santoni et al., 2023). These phenolic compounds have high antioxidant properties and various other biological activities, such as anti-cholesterol (Pratiwi et al., 2021), antibacterial (Muharni et al., 2021), and anti-inflammatory activities (Aulena et al., 2023). In addition, sungkai leaves also play a role in increasing immunity and act as immunomodulatory agents (Dillasamola et al., 2022).

(a) (b)

Figure 1. Sungkai plants (a) and sungkai leaves (b)

Several previous studies have demonstrated the antioxidant activity of various extracts. Ethanol extract of sungkai leaves from Central Bengkulu Regency, Bengkulu Province, showed an IC50 value of 50.83 mg/L for young sungkai leaves and 52.84 mg/L for old sungkai leaves (Okfrianti et al., 2022). Meanwhile, the ethyl acetate extract of sungkai leaves from Musi Banyuasin Regency, South Sumatra, has antioxidant activity with an IC50 value of 320 mg/L (Muharni et al., 2021). The antioxidant activity of the methanol extract of sungkai leaves from South Kalimantan showed an IC50 value of 63.977 mg/L (Sutomo et al., 2022). The antioxidant activity of sungkai leaves from Padang City, West Sumatra Province, showed an IC50 value of 13.589 mg/L for the methanol extract (Santoni et al., 2023).

Although various studies have been conducted on the antioxidant activity of sungkai leaf extracts from different regions, there is a lack of information that directly compares the potential antioxidant activity of the methanol and hexane fractions of sungkai leaves from the Tanah Datar region, which is characterized by volcanic soil. The liquid fractionation of plant extracts based on polarity is an effective method for separating and isolating different compounds from complex plant matrices. This technique exploits the varying affinities of compounds for solvents of different polarities, typically involving the use of solvents with increasing polarities to extract compounds sequentially.

For instance, Autor et al. 2022 described sequential extraction using solvents in the order of increasing polarity: n-hexane, dichloromethane, ethyl acetate, methanol, and water. This approach allows the separation of compounds based on their polarity, with non-polar compounds extracted first and polar compounds extracted last (Autor et al., 2022). One advantage of this method is its ability to concentrate specific compounds in different fractions, thereby focusing on specific active compounds with potential for higher and more targeted activity. Consequently, this study aimed to compare the antioxidant activity of hexane and methanol fractions of sungkai leaves from the Tanah Datar region to ascertain which fraction exhibits superior activity, thereby supporting the development of natural materials as an effective and selective source of antioxidants.

METHOD

Instruments and Materials

Instruments

Glassware, rotary evaporator (Buchi, Rotavapor® R-100, R-300) and UV-Vis spectrophotometer (ThermoFisher Scientific, GENESYS 30 Visible Spectrophotometer).

Materials

Sungkai leaf samples (*Peronema canesces* Jack) were collected from the Salak River region in Saruaso Village, Tanjung Emas District of Tanah Datar Regency. The extraction process utilized hexane, ethyl acetate, and methanol were used as solvents. Antioxidant tests involved DPPH and ascorbic acid, while total phenolic tests employed 20% sodium carbonate, Folin-Ciocalteu reagent, gallic acid, and distilled water.

Fractionation of Methanol Extract

A total of 20 g of the concentrated methanol extract was fractionated using hexane and ethyl acetate. The methanol extract was placed in a separatory funnel, and water and hexane were added at a ratio of 1:2, stirred gently until mixed, and allowed to separate into two phases. The methanol fraction was separated from the hexane fraction by opening the tap of the separatory funnel and collected in a glass Erlenmeyer flask. This process was repeated several times using the same solvent, until a clear hexane layer was obtained. In the same manner, fractionation was continued with an ethyl acetate solvent. The ethyl acetate fraction and remaining fraction (methanol) were collected (Pertiwi et al., 2023). concentrated all fractions (methanol, ethyl acetate, and n-hexane) using a rotary evaporator, weighed, and tested for antioxidant activity and total phenolic content.

Total Phenolic Content Test of Hexane Fraction and Methanol Fraction of Sungkai Leaves

Total phenolic content was assessed in accordance with the methodologies outlined by Salim et al. (2019) and Santoni et al. (2023). This procedure was applied to the fractions obtained from sungkai leaf fractionation using the Folin-Ciocalteu method (Salim et al., 2019; Santoni et al., 2023).

Calibration Curve Manufacturing of Gallic Acid Standard Solution

Gallic acid 0.01 g was dissolved in a 10 mL volumetric flask using methanol, resulting in a stock solution with a concentration of 1000 mg/L. The concentrations used were 20, 40, 60, 80, and 100 mg/L. Each concentration was pipetted into a 10 mL volumetric flask, with each solution (0.5 mL), followed by the addition of Folin-Ciocalteu reagrnt (0.5 mL) and allowed to stand for 5 min. Subsequently, 1 mL of a 20% Na2CO3 solution was added to each mixture. The mixture was then incubated for 120 min at room temperature. The absorbance of each sample was measured at a wavelength of 765 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific, GENESYS 30 Visible Spectrophotometer) at room temperature. Statistical analyses were conducted using linear regression based on triplicate measurements for each concentration. A calibration curve was constructed to establish the relationship between concentration and absorbance, thereby deriving at regression equation for the standard solution (Santoni et al., 2023).

Determination of the Total Phenolic Content of the Test Sample

A solution of the hexane and methanol fractions with a concentration of 10 mg/10 mL methanol was prepared, yielding a master solution with a concentration of 1000 mg/L. A test solution with a concentration of 500 mg/L was subsequently prepared. The test solution (0.5

mL) was pipetted into a 10 mL volumetric flask, followed by the addition of Folin-Ciocalteu phenol reagent (0.5 mL). After 5 min, 1 mL of a 20% Na2CO3 solution was added to the mixture. The solution was diluted to the desired concentration with distilled water. The mixture was incubated for 120 min at room temperature, and the absorbance of each sample was measured at a wavelength of 765 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific, GENESYS 30 Visible Spectrophotometer) at room temperature. The results were expressed as a percentage (%) equivalent to gallic acid, GAE (mg GAE/g dried extract or dried leaf) (Salim et al., 2019; Santoni et al., 2023).

Antioxidant Activity Test of Hexane Fraction and Methanol Fraction of Sungkai Leaf

The antioxidant activities of the hexane and methanol fractions of Sungkai leaves were evaluated using a modified procedure based on the method described by Ammaji et al. (2022). The assessment employed DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Ammaji et al., 2022).

Manufacturing of DPPH Solution

Four milligrams of DPPH powder were weighed and diluted to a final volume of 100 mL using methanol. The resulting 0.1 mM DPPH solution was stored in a sealed container and incubated at 40°C for 30 min (Ammaji et al., 2022).

Test Solution Manufacturing

The hexane and methanol fractions were each weighed to 1 mg and dissolved in methanol in a 10 mL volumetric flask to achieve a concentration of 100 mg/L. Five different test solution concentration were prepared: 80, 60, 40, 20, and 10 mg/L.

Manufacture and Testing of Ascorbic Acid Solution as Positive Control

Ascorbic acid (1 mg) was dissolved in methanol in a 10 mL volumetric flask to obtain a solution with a concentration of 100 mg/L. Concentrations of 1, 2, 3, 4, and 5 mg/L were used. The ascorbic acid solution was mixed with 2 mL and 3 mL of 0.1 mM DPPH solution, and the mixture was allowed to stand for 30 min. The absorbance of each sample was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific, GENESYS 30 Visible Spectrophotometer) at room temperature. Statistical analyses were conducted using linear regression based on triplicate measurements for each concentration. This procedure was conducted in a dark environment to prevent exposure to sunlight (Ammaji et al., 2022).

Antioxidant Activity Testing Test Sample

Antioxidant activity was assessed by adding 3 mL of 0.1 mM DPPH solution to 2 mL of the test solution at various concentrations. As a control, 2 mL methanol was added to 3 mL DPPH solution. The mixtures were kept in the dark, away from sunlight, for 30 min. The absorbance of each test and control solution was measured at a wavelength of 517 nm. Percentage inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \times 100\% \quad (1)$$

Information:

Ac = control absorbance value
As = sample absorbance value

Following the calculation of % inhibition, the IC50 value of each extract was determined using the regression equation (Rahmi Z J et al., 2023).

RESULTS AND DISCUSSION

Total Phenolic Content of Hexane Fraction and Methanol Fraction

Phenolic compounds constitute a diverse group of plant-derived substances known for their bioactive properties. The total phenolic content (TPC) is frequently employed in the qualimetric evaluation of complex samples analyses, as it is less labor-intensive than the quantification of individual phenolic compounds. This approach is particularly advantageous, given the identification of approximately 9000 plant phenolic substances to date. Notably, phenolic compounds can exist in both free and bound forms, influencing their extractability and interactions with cell wall components (Tarasov et al., 2023; Wu et al., 2022). Phenolic compounds are important molecules that serve as antioxidants, contributing to the inhibition of free radicals by donating hydrogen atoms to these radicals (Aryal et al., 2019).

This assay is based on the Folin-Ciocalteu reagent's capacity to oxidize the hydroxyl group (-OH) of phenolic compounds. Total phenolic content was assessed spectroscopically using gallic acid as the standard. Gallic acid was selected because is classified as a hydroxy benzoate derivative, which is a type of phenolic acid. The efficacy of phenolic acids in free radical inhibition is contingent upon the number and position of the hydroxyl and methoxy groups within their aromatic structures. Gallic acid, also known as 3,4,5-trihydroxybenzoic acid, is recognized as the most potent antioxidant among phenolic acids (Pérez et al., 2023). Furthermore, gallic acid is a stable natural phenolic compound that is more cost-effective than other (Hilma, 2018). This method is based on the principle of oxidizing phenolic compounds in a sample using the Folin–Ciocalteu reagent. This reagent comprises a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$).

Upon oxidation of phenols, the reagent is reduced to a combination of blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}) (Michiu et al., 2022). As illustrated in **Figure 2**, phenolic compounds can reduce phosphomolybdate compounds in Folin-Ciocalteu reagents, resulting in the formation of blue molybdenum complexes (Sami et al., 2019). This reduction process induces a color change from yellow to blue, and the intensity of this change is directly proportional to the concentration of phenolic compounds in the sample (Pérez et al., 2023). A higher concentration of gallic acid correlates with an increased absorbance value due to the more intense color produced, which is attributable to the substantial number of phenolic ions formed (Santoni et al., 2023).

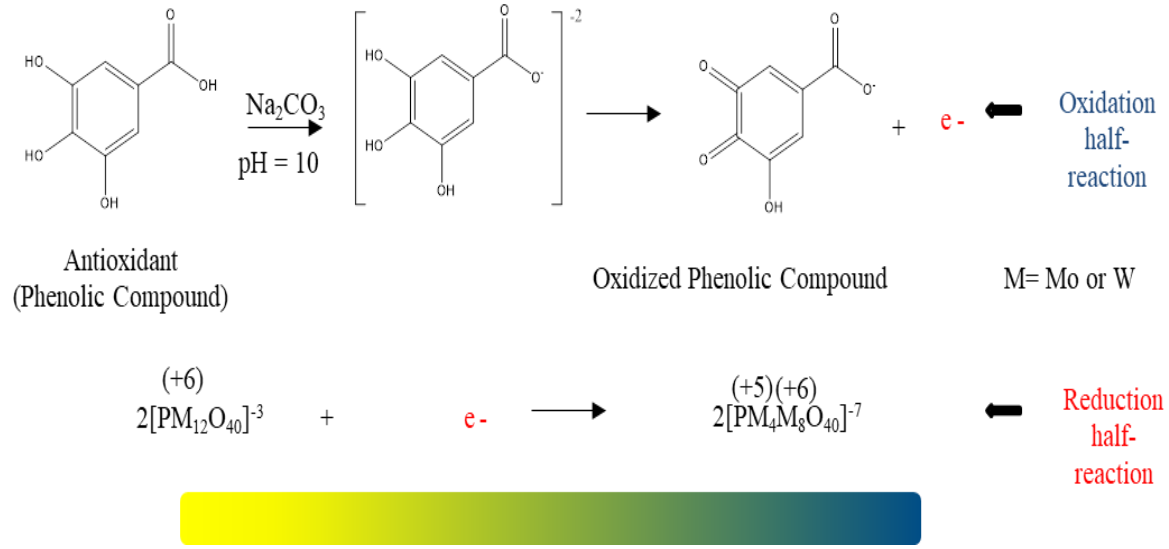

Figure 2. Redox reactions in the Folin-Ciocalteu test of metal complexes with phenolic compounds

Gallic acid changes color from yellow to blue after reacting with the Folin-Ciocalteu reagent in an alkaline medium. A sodium carbonate solution was used to adjust the pH to approximately 10, thus preventing excessive alkalinity. The reaction was allowed to proceed for two hours at room temperature to ensure completion, resulting in a stable blue color. This blue color can be observed at a wavelength of approximately 760 nm. The reducing capacity of phenolic compounds was quantified as gallic acid equivalents (GAE) (Pérez et al., 2023).

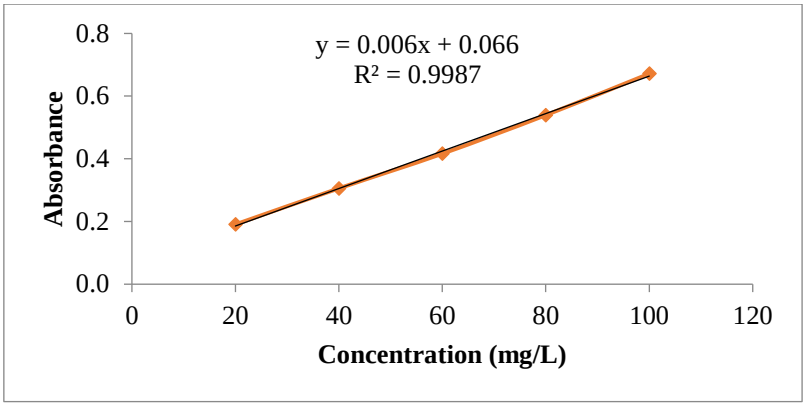

Figure 3. Gallic acid standard curve

The total phenolic content was determined based on the regression equation of the calibration curve of the standard gallic acid solution, as shown in **Figure 3.** Based on the results of this study, the total phenolic content of each fraction was 25.33 mg GAE/gram and 92.22 mg GAE/gram for the hexane and methanol fractions.

Table 1. Data on the total phenolic content of sungkai leaf fractions

No	Faction	Absorbance	Mg GAE/gram
1	Hexane	0.142	25.33
2	Methanol	0.343	92.22

As shown in **Table 1**, the methanol fraction had a higher TPC than the hexane fraction. This indicates that the methanol fraction contained many phenolic compounds that act as antioxidants (Aryal et al., 2019).

Antioxidant Activity of Hexane Fraction and Methanol Fraction of Sungkai Leaf

The antioxidant activities of the hexane and methanol fractions of the sungkai leaves were assessed using the DPPH method. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is widely used to evaluate the antioxidant capacities of various compounds and extracts. This method was selected because of its efficiency, simplicity, and cost-effectiveness for evaluating antioxidant activity (Christodoulou et al., 2022). The tests were conducted at various concentrations: 5, 10, 20, 40, and 80 mg/L for the methanol fraction and 10, 20, 40, 80, and 100 mg/L for the hexane fraction. Ascorbic acid served as a control, with concentrations ranging from 1 mg/L to 5 mg/L. Antioxidant activity was calculated based on the percentage of DPPH free radical inhibition by the antioxidant compounds. This principle relies on the reduction of DPPH free radicals, which are identified through a stable purple-colored compound. When DPPH, a stable free radical, reacts with antioxidants, it is reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH-H), resulting in a color change from purple to yellow or colorless. This color change is measured spectrophotometrically, typically at 517 nm, to quantify antioxidant activity (Baliyan et al., 2022; Flieger & Flieger, 2020; Muliasari et al., 2023).

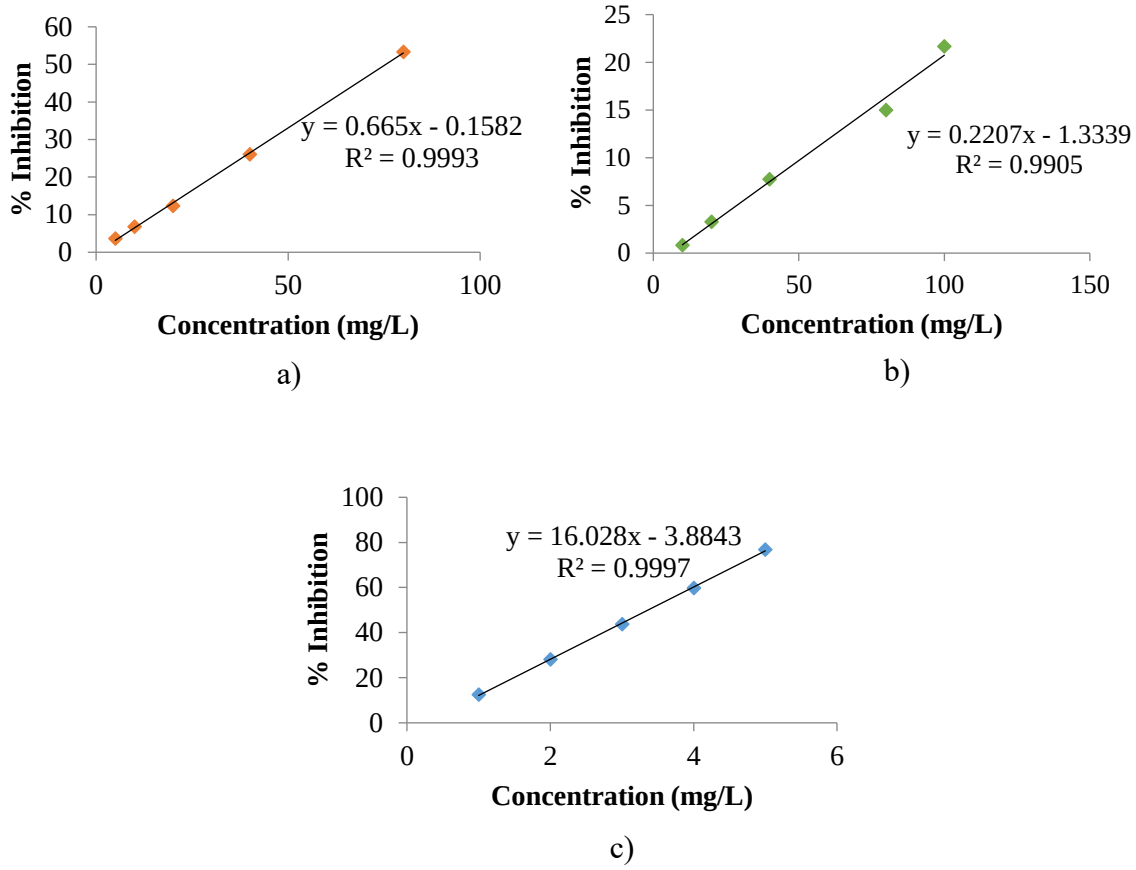

Figure 4. The sample concentration relationship curve with the percent inhibition of a) methanol fraction, b) hexane fraction, and c) ascorbic acid as control

Table 2. IC50 values of each fraction and ascorbic acid

No.	Sample	IC50 Value (mg/L)
1	Hexane	232,595
2	Methanol	75,425
3	Ascorbic acid	3,362

The antioxidant activity was measured based on the IC50 value, which is the concentration required to inhibit 50% of free radicals; the smaller the IC50 value, the higher the antioxidant activity of the sample (Peni Pindan et al., 2021).

Based on the graph shown in **Figure 4**, the increase in concentration was in line with the increase in the percentage of inhibition. This is because, at higher sample concentration, more free radicals are inhibited by antioxidant compounds. The IC50 values for antioxidant activity of each fraction are listed in Table 2. A sample was categorized as a very strong antioxidant if it had an IC50 value of <50 mg/L, moderate if it was between 50-100 mg/L and weak if it was >100 mg/L(Rahmi Z J et al., 2023). As shown in Table 2, the antioxidant activity of the methanol fraction was moderate, with an IC50 value between 50-100 mg/L, whereas the antioxidant activity of the hexane fraction was relatively weak, with an IC50 value of more than 100 mg/L.

In general, the contents of sungkai leaf extracts have been identified, including flavonoids, tannins, phenolics, and alkaloids (Peni Pindan et al., 2021). In a previous study by Santoni et al.(2023), phytochemical tests on three sungkai leaf extracts showed that the methanol extract contained flavonoids, phenolics, saponins, triterpenoids, and steroids, whereas the ethyl

acetate extracts contained flavonoids, phenolics, saponins, and steroids. Phytochemical tests of the hexane extracts showed only the presence of triterpenoid compounds and alkaloids (Santoni et al., 2023). According to the total phenolic test data from the two sungkai leaf fractions, the methanol fraction contained the highest concentration of phenolic compounds, indicating that this fraction possessed significant antioxidant activity.

Various categories of phenolic compounds such as flavonoids, tannins, and other phenolic derivatives have shown potential as antioxidants. The antioxidant activities of these compounds were augmented by an increase in the number of hydroxyl groups. Phenolic compounds contribute to the stabilization of radical species by donating hydrogen atoms or electrons to free radicals; thus, a higher flavonoid content in extracts is associated with increased antioxidant activity (Muharni et al., 2021). Phenolic compounds and their derivatives such as flavonoids and tannins have been extensively documented as potential reducers and inhibitors of free radicals. Furthermore, flavonoid compounds and tannins, which are classified as a class of phenolic compounds and generally possess antioxidant activity, provide synergistic effects that enhance the activity of each other(Khelouf et al., 2023; Sutomo et al., 2022).

Based on their structure, triterpenoid compounds with phenolic groups can exhibit antioxidant activity; however, not all triterpenoid compounds have this group. While alkaloid compounds can contribute their hydrogen atoms to free radicals, not all compounds in this group have antioxidant activity because of their diverse bioactivities, such as analgesic, anti-inflammatory, and anticancer effects (Hasan et al., 2022; Vanesa & Ikhsan, 2023). The difference in solvents used in the fractionation process affect the composition of the active compounds. Phenolic compounds are generally polar; therefore, they are more easily soluble in polar solvents such as methanol. Therefore, the methanol fraction tended to contain a higher total phenolic compound content and exhibited stronger antioxidant activity than the hexane fraction, which is more effective at extracting non-polar compounds such as some types of terpenoids and alkaloids that may not have dominant antioxidant activity.

In addition, the type and amount of metabolites present in plants are not only determined by genetic factors and solvent extraction but can also be influenced by the environmental conditions in which the plant grows. Volcanic soil can significantly influence the production of secondary metabolites in plants, thereby affecting their growth, development, and defense mechanisms. Volcanic ash deposition can disrupt soil surface hydrology and alter soil properties, which, in turn, affects plant metabolism (Saputra et al., 2022, 2023). The interaction between volcanic ash and organic matter can induce water repellency, affecting water infiltration and potentially altering plant-soil interactions (Saputra et al., 2023). These changes in soil conditions can trigger stress responses in plants, leading to modifications in the accumulation of primary and secondary metabolites (Salam et al., 2023). Interestingly, while volcanic ash can have negative effects on soil quality and plant growth, it can also contribute beneficial elements to the soil (Carrera-Beltrán et al., 2024).

CONCLUSION

Upon analysis of the conducted research, it is evident that this study has evaluated the antioxidant potential of hexane and methanol fractions derived from the leaves of *Paronema canescens* Jack, commonly known as sungkai, sourced from Tanah Datar Regency, West Sumatra, Indonesia. The methanol fraction exhibited a significantly higher total phenolic content, measured at 92.22 mg GAE/g, in contrast to the hexane fraction, which recorded 25.33 mg GAE/g. Furthermore, the methanol fraction demonstrated superior antioxidant activity, with an IC50 value of 75.425 mg/L, whereas the hexane fraction displayed

comparatively weaker antioxidant activity, with an IC50 value of 232.595 mg/L. The enhanced antioxidant activity of the methanol fraction is likely attributable to its elevated concentration of phenolic compounds, including flavonoids and tannins, known for their potent antioxidant properties. The synergistic interaction of these compounds may further augment the overall antioxidant capacity of the methanol fraction.

RECOMMENDATIONS

In the next study, the research topic to be carried out is the analysis of the antioxidant activity of the compounds contained in each of the fractions of sungkai leaves, both experimentally (compound isolation) and computationall, including molecular docking analysis. An obstacle that may be faced is the difficulty in identifying the target compound in isolation.

ACKNOWLEDGEMENTS

We thank the Faculty of Mathematics and Natural Science, Universitas Andalas (no. 06/UN.16.03.D/PP/FMIPA/2024) for their support and financial assistance, which enabled successful implementation of this study.

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