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The Effect of Extraction Methods on Antioxidant Activity of *Acacia auriculiformis* Stem Bark Ethanol Extract

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ABSTRACT

Introduction: *Acacia auriculiformis was recognized as a member of the Fabaceae family, and its stem bark exhibited pharmacological potential. The stem bark of Acacia auriculiformis has been traditionally used by native Australians to treat rheumatism.* ***Aims***: *This study aimed to identify the chemical compounds and determine the effect of extraction methods on the antioxidant activity of the 70% ethanol extract of Acacia auriculiformis stem bark using the DPPH assay.* ***Method***: *Antioxidant activity was expressed as the 50% scavenging concentration (SC_{50}) and the Antioxidant Activity Index (AAI). The 70% ethanol extract of Acacia auriculiformis stem bark demonstrated antioxidant activity qualitatively through the dot-blot staining assay.* ***Result***: *Phytochemical screening results showed that the Acacia auriculiformis stem bark obtained through maceration and reflux contained alkaloids, phenolics, flavonoids, and saponins. The antioxidant assay indicates antioxidant activity using the DPPH method in the stem bark extract of Acacia auriculiformis obtained through maceration and reflux, with SC_{50} values of 2.08 µg/mL and 2.74 µg/mL, respectively, and AAI values of 19.20 and 14.61. Data analysis reveals no significant difference between the two extraction methods in terms of antioxidant activity, as determined by Tukey's test ($p \geq 0.05$).* ***Conclusion***: *These findings indicate that both 70% ethanol extracts obtained through maceration and reflux exhibited antioxidant activity.*

KEYWORDS: *Acacia auriculiformis, stem bark, extraction, antioxidant, DPPH.*

INTRODUCTION

Acacia auriculiformis A.Cunn ex Benth. belongs to the family Fabaceae and is native to Australia, Papua New Guinea, and Indonesia. This plant was first introduced to India in 1946 in West Bengal. Acacia auriculiformis has synonyms such as Acacia auriculiformis A.Cunn ex Benth., orth. Var., Acacia moniliformis Griseb, and Racosperma auriculiformae (A.Cunn ex Benth.) Pedley (Rangra et al., 2019). The plant is traditionally used as medicine to treat various diseases, such as eye disorders, inflammation, malaria, and skin conditions, including itching, allergy, and rashes (Sharma et al., 2017). The bark of Acacia auriculiformis A.Cunn ex Benth. has been reported to possess potential as a

tanning agent (Mutiar et al., 2018), wound healing (Singh & Sharma, 2014), rheumatism treatment (Girijashankar, 2011), antibacterial, antidiabetic (Johnson et al., 2021), antioxidant (Singh et al., 2007), analgesic and anti-inflammatory, as well as anticancer properties (Ahmadu et al., 2019; Kaur et al., 2002).

In this study, we report the phytochemical constituents and the effect of extraction methods on the antioxidant activity of the 70% ethanol bark extract of Acacia auriculiformis A.Cunn ex Benth. using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The findings of this study are expected to serve as a foundational step toward the discovery and development of novel antioxidant compounds derived from natural sources.

METHODS

Materials and Instruments

Technical grade 70% ethanol is used for maceration and reflux purposes. Analytical grade chemicals were used for phytochemical identification and antioxidant activity assays: ascorbic acid (Merck), DPPH (2,2-diphenyl-1-picrylhydrazil) (Sigma Aldrich), methanol (Merck), nitric acid (Merck), bismuth (III) nitrate (Merck), KI (Merck), $FeCl_3$ (Merck), acetic acid (glacial) (Merck), H_2SO_4 (Merck), magnesium powder (Merck), HCl (Merck), TLC plate (Merck).

Sample Collection and Simplisia Preparation

The stem bark of *Acacia auriculiformis* was collected from Kolaka District, Kolaka Regency, Southeast Sulawesi Province, Indonesia, in June 2025. Sampling was carried out in the morning. Wet sorting was then performed to remove dirt and foreign materials from the fresh plant samples. The samples were subsequently washed under running water to eliminate any remaining impurities. Drying was carried out without direct exposure to sunlight. The drying process aimed to reduce the moisture content, thereby preventing mold growth and enzymatic reactions that could decrease sample quality. After drying, dry sorting was conducted to remove any remaining contaminants or undesirable plant parts. Finally, the stem bark was powdered to facilitate subsequent processes, such as extraction (Djuwarno et al., 2022).

The determination of plant material was carried out at the Laboratory of Traditional Medicine Raw Materials, National Research and Innovation Agency, through E-Layanan Sains-BRIN. The plant was identified as *Acacia auriculiformis* A.Cunn. ex Benth. (Fabaceae), locally known as akasia.

Extraction

Extraction by maceration was carried out by weighing 100 g of powdered stem bark and placing it into a maceration vessel. Subsequently, 1.5 L of 70% ethanol was added. The mixture was kept for 3 × 24 hours, protected from sunlight, with occasional stirring. The resulting mixture was then filtered and concentrated using a rotary evaporator to obtain a thick extract. The concentrated extract was weighed to determine its yield (Sari & Putra, 2018; Doloking et al., 2022).

Extraction using the reflux method was carried out by weighing 100 g of powdered stem bark and placing it into a round-bottom flask. Subsequently, 1.5 L of 70% ethanol was added, and the mixture was heated at 60 °C for 3 hours. The resulting mixture was then filtered and concentrated using a rotary evaporator to obtain a thick extract. The concentrated extract was weighed to determine its yield (Jubaidah et al., 2024).

Phytochemical Screening

The extract solutions obtained from maceration and reflux were placed into test tubes, followed by the addition of specific reagents to identify the classes of chemical compounds.

Alkaloid test. The presence of alkaloids was indicated by the formation of an orange to reddish brown precipitate after adding 2 drops of Dragendorff's reagent to the extract solution (Sumarni et al., 2019)..

Phenolic test. The presence of phenolic compounds was indicated by the formation of green, red, blue, purple, or black coloration after the addition of several drops of 1% $FeCl_3$ solution to the extract solution (Sumarni et al., 2019)..

Triterpenoid and steroid test. The presence of triterpenoids was indicated by the appearance of red or purple coloration, whereas the presence of steroids was indicated by green or blue coloration after the addition of 3 drops of Liebermann–Burchard reagent to the extract solution (Sumarni et al., 2019)..

Flavonoid test. The presence of flavonoids was indicated by the formation of red, green, yellow, or orange coloration after the addition of 2 mg of Mg powder and 3 drops of concentrated HCl to the extract solution (Sumarni et al., 2019)..

Saponin test. The presence of saponins was indicated by the formation of stable foam with a height of 1–3 cm after vigorous shaking of the extract solution, which persisted for 5 minutes after the addition of 1 drop of concentrated HCl (Sumarni et al., 2019).

Antioxidant Activity Assay Using The DPPH Method

The antioxidant activity test was carried out qualitatively and quantitatively according to a previous method with slight

modifications. Qualitatively (dot-blot staining on TLC plate), 5 μL of a series of extract solutions with eight concentrations in 96% ethanol was spotted onto a TLC plate, which was then dipped into a DPPH solution (0.5 mM in 96% ethanol) and immediately removed. The plate was incubated for 30 minutes in the dark at room temperature. The final sample concentrations on the plate ranged from 100 to 0.8 μL/spot. The presence of radical scavenging activity was indicated by the formation of a yellow zone against a purple background on the TLC plate (Sabandar et al., 2023).

Quantitatively (spectrohotometric method), a total of 2 mL of the sample solution was mixed with 2 mL of 40 μg/mL DPPH solution. The mixture was homogenized and then incubated for 30 minutes in the dark. Absorbance was measured at a maximum wavelength of 515 nm. The final concentration of all solutions in the mixture ranged from 100 to 0.8 μg/mL. Ascorbic acid solution was used as a reference standard. Antioxidant activity (% radical scavenging activity, %RSA) was calculated using the following equation:

$$\%\text{RSA} = \frac{\text{Absorbance}_{\text{blank}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{blank}}} \times 100\%$$

(Muzdalifa & Jamal, 2019; Rohmah, 2022)

The blank absorbance was defined as the absorbance of the DPPH solution (40 μg/mL), whereas the sample absorbance was calculated as the absorbance of the sample combined with the DPPH solution minus the absorbance of the sample solution without DPPH. This procedure was performed to eliminate the absorbance of compounds in the extract that also absorb light at the DPPH wavelength (Yuniar et al., 2025).

The extract concentration required to scavenge 50% of DPPH radicals (SC_{50}) was determined based on the obtained %RSA values. The 50% radical scavenging concentration (SC_{50}) values of the 70% ethanol stem bark extract obtained by maceration, the 70% ethanol stem bark extract obtained by reflux, and the positive control (ascorbic acid) were determined using GraphPad Prism 5 software (GraphPad Inc., California, USA) (Sabandar et al., 2020).

The Antioxidant Activity Index (AAI) is classified as weak (AAI < 0.5), moderate (AAI 0.5–1.0), strong (AAI 1.0–2.0), and very strong (AAI > 2.0). The AAI was calculated using the following equation:

$$\text{AAI} = \frac{\text{Final DPPH concentration } (\mu\text{g/mL})}{\text{Scavenging Concentration}_{50}\ (\mu\text{g/mL})}$$

(Nayaka et al., 2023)

Data Analysis

The data from the tests were expressed as mean ± standard deviation of three replicates. Statistical analysis was performed using GraphPad Prism 5

Table 1. Extraction results of *Acacia auriculiformis* A.Cunn. ex Benth. stem bark

Extraction Method	% Yield
Maceration extract	40.7
Reflux extract	28.6

software. Differences among groups were compared using Analysis of Variance (ANOVA) followed by Tukey's post hoc test ($p < 0.05$).

RESULTS AND DISCUSSION

Extraction

Maceration and reflux of *Acacia auriculiformis* stem bark using 70% ethanol produced a brown extract with the yield percentage shown in Table 1. Yield refers to the ratio of extract obtained to the initial amount of simplisia. The extraction using the maceration method produced a higher yield compared to the reflux method. The yield percentage indicates the amount of extract obtained from the extraction process and is also related to the active compounds present in the sample. The higher the yield, the greater the content of active compounds in the sample (Pawarti et al., 2023).

The yield is influenced by several factors, such as the particle size of the simplisia, the type of solvent, the solvent-to-sample ratio, temperature, and extraction time. Smaller particle sizes increase the contact surface area between the sample and the solvent, enhancing the diffusion of compounds from within the cells into the solvent and increasing the amount of compounds extracted (Asworo & Widwiastuti, 2023). The type of solvent used affects which compounds can be extracted, based on the principle of solubility, "like dissolves like," where polar solvents dissolve polar compounds and nonpolar solvents dissolve nonpolar compounds (Prasetya et al., 2020). Increasing the amount of solvent can enhance the yield during the extraction process (Rifai et al., 2018). A larger volume of solvent generally produces a higher yield (Kusuma & Aprileili, 2022).

Raising the temperature can increase solubility and diffusion of compounds, thereby improving yield. However, excessive temperature may lead to degradation of thermolabile compounds, reducing the yield (Rifkia & Prabowo, 2020). The duration of maceration affects the yield obtained. Longer extraction times allow prolonged contact between the sample and solvent, increasing the amount of dissolved compounds (Pamudi et al., 2021).

Phytochemical Screening

Phytochemical screening is a qualitative analytical method used to identify the classes of active compounds present in a sample. The screening was performed by adding specific identification reagents to separate test tubes containing the extract solutions to determine the chemical compound classes in the extract. The results

Table 2. Phytochemical screening results of *Acacia auriculiformis* A.Cunn. ex Benth. stem bark with various extract

Chemical constituents	Maceration extract	Reflux extract
Alkaloids	+	+
Phenolics	+	+
Flavonoids	+	+
Triterpenoids and steroids	–	–
Saponins	+	+

(+) presence of secondary metabolites
(–) absence of secondary metabolites

Table 3. DPPH radical scavenging activity of the 70% ethanol extract of *Acacia auriculiformis* A.Cunn. ex Benth. stem bark with various extraction method

Sample	%RSA (at 100 μg/mL)	SC_{50} (μg/mL)	AAI
Maceration extract	93.38 ± 2.26	2.08	19.20
Reflux extract	89.24 ± 0.82	2.74	14.61
Ascorbic acid	96.76 ± 1.02	2.51	15.93

of the phytochemical screening are presented in Table 2. The screening of the 70% ethanol stem bark extract of *Acacia auriculiformis* A.Cunn. ex Benth., obtained through both maceration and reflux methods, revealed the presence of the same chemical compounds, namely alkaloids, phenolics, flavonoids, and saponins.

Antioxidant Activity Using The DPPH Method

The 70% ethanol stem bark extract of *Acacia auriculiformis* A.Cunn. ex Benth., obtained through maceration and reflux, exhibited qualitative antioxidant activity in scavenging DPPH radicals, compared to ascorbic acid as the positive control. The DPPH scavenging activity was evaluated at concentrations ranging from 100 to 0.8 μg/spot, as shown in Figure 1. Higher sample concentrations produced larger yellow zones on the TLC plate. The results of the DPPH dot-blot staining assay indicated that the 70% ethanol stem bark extract of *Acacia auriculiformis* obtained through both maceration and reflux was capable of scavenging DPPH radicals.

The 70% ethanol stem bark extract of *Acacia auriculiformis* A.Cunn. ex Benth., obtained through maceration and reflux, exhibited quantitative antioxidant activity, as shown in Table 3. However, the activity of both extracts was still lower than that of the positive control (ascorbic acid), as illustrated in Figure 2. The SC_{50} represents the concentration at which the sample can scavenge or reduce 50% of free radical activity. Its value is influenced by the analytical method, including the concentrations used in the experiment. In contrast, the Antioxidant Activity Index (AAI) remains constant despite variations

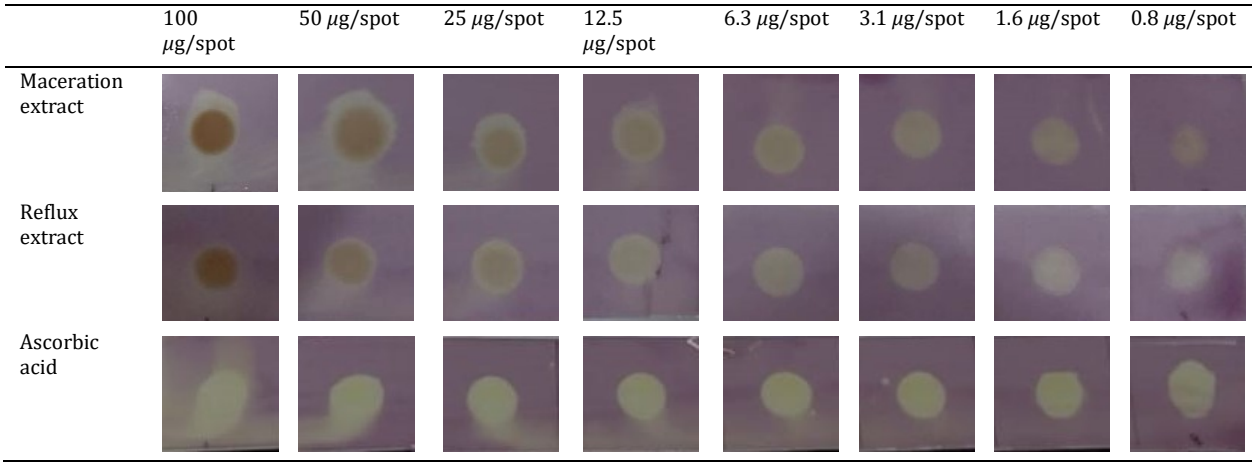

Figure 1. Dot-blot DPPH staining assay results of the ethanol extract of *Acacia auriculiformis* A.Cunn. ex Benth. stem bark

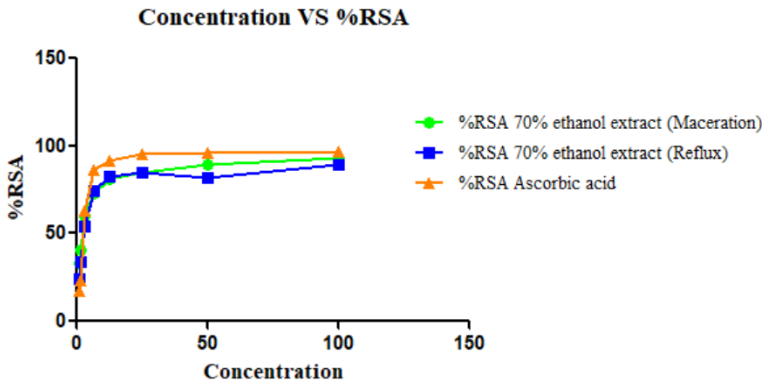

Figure 2. Antioxidant activity of the ethanol extract of *Acacia auriculiformis* A.Cunn. ex Benth. stem bark

in free radical concentration. Therefore, expressing antioxidant activity as the AAI is considered more appropriate (Nayaka et al., 2023).

Based on the AAI values, the 70% ethanol extract obtained through maceration, the 70% ethanol extract obtained through reflux, and ascorbic acid (positive control) all exhibited very strong antioxidant activity. Analysis using Tukey's test indicated no significant difference ($p \geq 0.05$) between the extraction methods (maceration and reflux) regarding antioxidant activity. This indicates that the extraction method did not affect the antioxidant activity of the 70% ethanol stem bark extract of *Acacia auriculiformis* A.Cunn. ex Benth. These findings suggest that the 70% ethanol stem bark extract of *Acacia auriculiformis* has potential as a natural source of antioxidants that warrants further development.

CONCLUSION

As a result of this study, the 70% ethanol extract of *Acacia auriculiformis* A.Cunn. ex Benth. stem bark obtained through maceration had a higher yield (40.7%) compared to the 70% ethanol extract obtained through reflux (28.6%). Both the maceration and reflux extracts contained alkaloids, phenolics, flavonoids, and

saponins. Furthermore, the 70% ethanol stem bark extracts obtained by both methods exhibited antioxidant activity. The extraction method (maceration or reflux) did not significantly affect the antioxidant activity of the 70% ethanol stem bark extract of *A. auriculiformis*. These findings suggest that the 70% ethanol stem bark extract of *A. auriculiformis* has potential as a natural source of antioxidants that warrants further development.

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