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Comparative Evaluation of Maceration, Reflux, Ultrasound-Assisted Extraction and Microwave-Assisted Extraction on the Antioxidant Activity of *Leea aequata* L. Leaves via DPPH Assay

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ABSTRACT

Introduction*: Natural antioxidants from medicinal plants have gained attention due to their ability to neutralize free radicals and reduce oxidative stress-related disorders. Leea aequata L. leaves are traditionally used in North Sumatra, Indonesia, and are reported to contain secondary metabolites with potential antioxidant activity.* ***Aims****: This study aimed to compare the antioxidant activity of Leea aequata L. leaf extracts obtained by maceration, reflux, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) using the DPPH radical-scavenging assay.* ***Methods:*** *Powdered dried leaves of Leea aequata L. were extracted using 96% ethanol through four extraction techniques. Basic physicochemical characterization was performed to ensure the quality of the dried plant material, while phytochemical screening was conducted to identify major secondary metabolites. Antioxidant activity was determined using the DPPH radical scavenging assay and expressed as IC_{50} values.* ***Results:*** *The extraction methods produced different yields and antioxidant activities. MAE gave the highest extraction yield (29.5%), followed by maceration (26.0%), UAE (20.0%), and reflux (11.0%). Phytochemical screening revealed the presence of flavonoids, phenolics, tannins, saponins, alkaloids, and steroids/triterpenoids. All extracts demonstrated very strong antioxidant activity, with IC_{50} values below 50 ppm. Among the tested extracts, MAE exhibited the strongest antioxidant activity, with the lowest IC_{50} value of 12.586 ± 0.04 ppm, followed by UAE (29.786 ± 0.05 ppm), maceration (37.656 ± 0.06 ppm), and reflux (40.581 ± 0.07 ppm).* ***Conclusion:*** *MAE was the most effective method for obtaining L. aequata leaf extract with strong antioxidant activity, as indicated by the strongest DPPH radical-scavenging activity.*

KEYWORDS*:* Antioxidant activity, DPPH assay, *Leea aequata* L., maceration, microwave-assisted extraction, reflux, ultrasound-assisted extraction.

INTRODUCTION

Free radicals are unstable, highly reactive species that damage proteins and DNA, contributing to aging, cancer, and other degenerative diseases (Widiasriani et al., 2024; Labagu et al., 2022). Antioxidants counteract this damage by donating electrons to neutralize free radicals

(Rustaman et al., 2023). Synthetic antioxidants such as BHA, BHT, and TBHQ raise safety concerns with long-term use (Irfan et al., 2022), prompting growing interest in natural antioxidant sources.

Plants contain secondary metabolites — flavonoids, alkaloids, tannins, and phenolics — that scavenge free radicals through hydrogen or electron donation (Tumilaar et al., 2024; Chaudhary et al., 2023). *Leea aequata* L. (Vitaceae), locally called Titanus leaf, is a traditional medicinal plant rich in phenolics, flavonoids, saponins, tannins, glycosides, and steroids (R. Bangar, Ningsih, Kartasasmita, & Insanu, 2024; Mostofa et al., 2024). Previous studies confirm its antioxidant potential: Hossain et al. (2024) linked its phenolic and flavonoid content to DPPH scavenging activity; Sihotang et al. (2025) reported an IC_{50} of 39.484 ppm, classified as strong, for its 96% ethanol macerate; and Waruwu (2025) found the ethyl acetate fraction more active (IC_{50} 11.309 µg/mL), attributed to its selectivity for phenolics and flavonoids (Sihotang, Bangar, Kaban, Sembiring, & Harahap, 2025; Waruwu, Bangar, Kaban, & Sembiring, 2025). These results show that extraction method and solvent type strongly affect antioxidant capacity.

Because extraction efficiency depends on temperature, time, and solvent-sample contact (Calderón-Oliver & Ponce-Alquicira, 2021), choosing an appropriate method is essential for optimal recovery of antioxidant compounds from Titanus leaves. Antioxidant activity is commonly assessed using the simple, rapid, and sensitive DPPH assay (Putri & Mahfur, 2023).

However, existing studies on *L. aequata* have examined single extracts or fractions without systematically comparing how different extraction mechanisms affect antioxidant recovery (Mungwari, King'ondu, Sigauke, & Obadele, 2025). This study therefore compared four mechanistically distinct methods: maceration (passive diffusion, low temperature); reflux (heat-assisted, continuous solvent contact) (Kumar et al., 2023); ultrasound-assisted extraction (acoustic cavitation enhancing solvent penetration) (Yusoff, Mat Taher, Rahmat, & Chua, 2022); and microwave-assisted extraction (dielectric heating promoting cell rupture) (López-Salazar, Camacho-Díaz, Ocampo, & Jiménez-Aparicio, 2023), to identify which mechanism most effectively enhances the DPPH radical-scavenging activity of *L. aequata* leaf extracts, an aspect that remains poorly characterized to date.

METHODS

Materials

The plant material used in this study was the leaves of *Leea aequata* L., locally known as daun titanus or daun tetanus, collected

from Patumbak District, Deli Serdang Regency, North Sumatra, Indonesia.

The chemicals used in this study included ethanol 96% (OneMed®), methanol p.a. (Merck®), distilled water (Brataco®), DPPH (2,2-diphenyl-1-picrylhydrazyl) (Himedia®), quercetin standard (Merck®), ferric chloride ($FeCl_3$) (Merck®), concentrated hydrochloric acid (HCl) (Merck®), sulfuric acid (H_2SO_4) (Smartlab®), chloroform (Smartlab®), acetic anhydride (Merck®), sodium hydroxide (NaOH), gelatin 1% (Merck®), sodium chloride (NaCl) (Brataco®), magnesium powder (Merck®), Mayer reagent (Nitra Kimia®), Dragendorff reagent (Nitra Kimia®), and Wagner reagent (Nitra Kimia®). All chemicals used were of analytical grade.

The instruments employed included UV–Visible spectrophotometer (T60®), rotary evaporator (IKA® HB Eco), analytical balance (Joil®), microwave oven (Samsung®), reflux apparatus equipped with heating mantle, bactosonic apparatus, water bath (Memmert®), magnetic stirrer, micropipettes (Dragon Lab®), quartz cuvettes, blender, mesh no. 40 sieve, and standard laboratory glassware such as volumetric flasks, beakers, measuring cylinders, and test tubes (Pyrex® and Iwaki®).

Plants Identification

The plant material used in this study consisted of Titanus leaves collected from Patumbak District, Deli Serdang Regency, North Sumatra, Indonesia, in September 2024. The plant material was authenticated at Herbarium Medanense, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. The identification process was conducted based on morphological characteristics, including leaf shape, stem morphology, and taxonomic classification, to ensure the authenticity and validity of the plant material used in this study (Maurya, Prakash, Shukla, & Shukla, 2025).

Sample Preparation

Fresh leaves of *Leea aequata* L. were manually sorted to remove damaged and contaminated material, then washed thoroughly under running water. The leaves were dried in a drying cabinet at controlled temperature without direct exposure to sunlight to prevent degradation of thermolabile compounds. The dried leaves were pulverized using a blender and sieved through mesh no. 40 to obtain homogeneous dried plant material powder (Waruwu et al., 2025).

Characterization of Dried Plant Material

Characterization of dried plant material was performed by determining loss on drying, moisture content, water-soluble

extractive value, ethanol-soluble extractive value, total ash content, and acid-insoluble ash content according to the Indonesian Herbal Pharmacopoeia standards. These parameters were evaluated to ensure the quality, purity, and stability of the plant material used in this study (Silalahi et al., 2025). All characterization measurements were performed in triplicate (n = 3), and the results were expressed as mean ± standard deviation (SD).

Extraction

Maceration Method

A total of 500 g of powdered dried plant material was immersed in 5 L of 96% ethanol with a solvent-to-material ratio of 1:10 (w/v). The extraction process was carried out at room temperature for 5 days with occasional stirring every 24 h. The obtained filtrate was filtered and concentrated using a rotary evaporator at 40–50°C, followed by evaporation on a water bath until a viscous extract was obtained (Kumar et al., 2023).

Reflux Method

A total of 200 g of dried plant material in powdered form was placed into a round-bottom flask and extracted with 2 L of 96% ethanol, maintaining a sample-to-solvent ratio of 1:10 (w/v). The extraction process was performed under reflux at 70°C for 2 h. Upon completion, the extract was allowed to cool to room temperature before being filtered through Whatman No. 1 filter paper. The resulting filtrate was concentrated using a rotary evaporator at 40–50°C, followed by further evaporation in a water bath until a viscous crude extract was obtained (Hidayati, Maghfiroh, & Safitri, 2023; Nguyen et al, 2026).

Ultrasound-Assisted Extraction (UAE) Method

A total of 250 g of powdered dried plant material was extracted with 2.5 L of 96% ethanol at a ratio of 1:10 (w/v). Prior to ultrasonication, the mixture was stirred using a magnetic stirrer for 30 min to improve solvent penetration. Ultrasonication was performed at a frequency of 40 kHz for 30, 45, and 60 min, respectively. The filtrate obtained was concentrated using a rotary evaporator at 40–50°C and evaporated further using a water bath until a concentrated extract was produced (Gavrila et al., 2022; Omer, Choo, Ashokkumar, & Yusof, 2022).

Microwave-Assisted Extraction (MAE)

As much as 200 g of powdered dried plant material was mixed with 2 L of 96% ethanol using a solvent-to-material ratio of 1:10 (w/v). Extraction was performed using a microwave oven at 300 W for 3, 5, 7, 9, 11, and 13 min. The extract was filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator at 40°C and 100 rpm until a viscous extract was obtained (García-Sarrió, Sanz, Palá-Paúl, Díaz, & Soria, 2023).

Phytochemical Screening

Phenolic Test

A total of 0.1 g of extract was treated with 3–4 drops of 5% $FeCl_3$ solution. The appearance of green, blue-green, blue, or black coloration indicated the presence of phenolic compounds (Hasan et al., 2026).

Flavonoid Test

As much as 0.1 g of extract was mixed with several drops of lead acetate solution [$Pb(CH_3COO)_2$]. The formation of yellow coloration indicated the presence of flavonoids (Hasan et al., 2026).

Tannin Test

A total of 0.1 g of extract was treated with $FeCl_3$ solution. The appearance of dark green or bluish-black coloration indicated the presence of tannins (Hasan et al., 2026).

Saponin Test

As much as 0.1 g of extract was mixed with 10 mL distilled water and shaken vigorously for 10 s. The mixture was allowed to stand for 10 min, followed by the addition of 1 mL 2 N HCl. Stable foam formation indicated the presence of saponins (Hasan et al., 2026).

Alkaloid Test

A total of 0.1 g of extract was dissolved in 1 mL concentrated HCl and divided into three test tubes. Mayer, Dragendorff, and Wagner reagents were added separately. The formation of orange-red precipitate with Dragendorff reagent, white precipitate with Mayer's reagent, or brown precipitate with Wagner reagent indicated the presence of alkaloids (Hasan et al., 2026).

Steroid and Triterpenoid Test

A total of 0.1 g of extract was dissolved in 2–3 mL chloroform, followed by the addition of 10 drops of acetic anhydride and 2–3 drops of concentrated H_2SO_4 (Liebermann–Burchard reagent). Blue-green coloration indicated steroids, while red-purple coloration indicated triterpenoids (Hasan et al., 2026; Oktavia & Sutoyo, 2021).

Antioxidant Activity

DPPH Method

The antioxidant activity of *Leea aequata* L. leaf extract was evaluated using the DPPH radical-scavenging assay, as described by Gulcin & Alwasel (2023). The extract solutions obtained by maceration, reflux, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) were prepared at concentrations of 4, 8, 16, 32, and 64 ppm. A total of 4 mL of each extract solution was mixed with 1 mL of freshly prepared 0.4 mM DPPH solution. The mixtures were homogenized and incubated in the dark at room temperature for 30 min to minimize DPPH photodegradation. The absorbance was subsequently measured at 517 nm using a UV–Visible spectrophotometer. A control solution containing 4 mL of methanol and 1 mL of 0.4 mM DPPH solution was prepared under the same conditions. All measurements were

performed in triplicate (n = 3) (Aparna & Hema, 2022).

Determination of Maximum Wavelength

The maximum absorption wavelength of DPPH was determined using a control solution consisting of 4 mL of methanol and 1 mL of 0.4 mM DPPH solution. The solution was homogenized and scanned using a UV–Visible spectrophotometer over the wavelength range of 500–525 nm. The maximum absorbance was observed at 517 nm, which was subsequently used for all antioxidant activity measurements (Aparna & Hema, 2022).

Preparation and DPPH Assay of Quercetin Standard Solution

A total of 25 mg of quercetin was accurately weighed and dissolved in 96% ethanol in a 25 mL volumetric flask to obtain a 1000 ppm stock solution. Subsequently, 2.5 mL of the stock solution was transferred into a 25 mL volumetric flask and diluted with 96% ethanol to obtain a 100 ppm working solution. Serial concentrations of 4, 8, 16, 32, and 64 ppm were then prepared from the working solution. A total of 4 mL of each quercetin standard solution was mixed with 1 mL of 0.4 mM DPPH solution, homogenized, and incubated in the dark at room temperature for 30 min. The absorbance was subsequently measured at 517 nm using a UV–Visible spectrophotometer, and all measurements were performed in triplicate (Aparna & Hema, 2022; Saputri, Susiani, & Asvia, 2023).

Preparation and DPPH Assay of Vitamin C Standard Solution

A total of 25 mg of vitamin C was accurately weighed and dissolved in 96% ethanol in a 25 mL volumetric flask to obtain a 1000 ppm stock solution. Subsequently, 2.5 mL of the stock solution was transferred into a 25 mL volumetric flask and diluted with 96% ethanol to obtain a 100 ppm working solution. Serial concentrations of 4, 8, 16, 32, and 64 ppm were prepared from the working solution. A total of 4 mL of each vitamin C standard solution was mixed with 1 mL of 0.4 mM DPPH solution. The mixtures were homogenized and incubated in the dark at room temperature for 30 min, after which absorbance was measured at 517 nm using a UV–Visible spectrophotometer. All measurements were performed in triplicate (Saputri et al., 2023).

Data Analysis

The percentage inhibition of DPPH radicals was calculated using the following equation:

$$\% \text{ Inhibision} = \frac{\text{Blank Absorbance} - \text{Sample Absorbance}}{\text{Blank Absorbance}} \times 100\%$$

Where blank Absorbance is the absorbance of the blank solution. Sample Absorbance is the absorbance of the sample solution.

Table 1. Characterization parameters of *Leea aequata* L. leaf dried plant material

Parameter	Result
Loss on drying (% w/w)	6.46 ± 0.16
Moisture content (% v/w)	7.66 ± 0.03
Water-soluble extractive (% w/w)	1.10 ± 0.15
Ethanol-soluble extractive (% w/w)	19.87 ± 0.63
Total ash content (% w/w)	5.03 ± 0.42
Acid-insoluble ash (% w/w)	0.90 ± 0.19

The IC_{50} value was determined from the linear regression equation obtained by plotting sample concentration against percentage inhibition. Antioxidant activity was classified based on IC_{50} values as follows: IC_{50} < 50 ppm (very strong), 50–100 ppm (strong), 100–150 ppm (moderate), 150–200 ppm (weak), and 200–250 ppm (very weak) (Yudistianik & Sinthary, 2025; Bangar, Saraswati, & Waruwu, 2026; Kaban, Bangar, Agustan, Sihotang, & Gaol, 2026).

RESULTS AND DISCUSSION

Plant Identification

The sample was confirmed as *Leea aequata* L. (Vitaceae) and deposited at Herbarium Medanense (voucher no. 2819/MEDA/2024), classified under Plantae, Spermatophyta, Dicotyledonae, and Vitales, confirming the taxonomic authenticity required for reproducible phytochemical and antioxidant research (Maurya et al., 2025).

Characteristics of Dried Plant Material

The dried leaves were obtained after three days of drying, then pulverized and sieved through mesh no. 40 to yield a homogeneous powder suitable for extraction.

Table 2. Extraction yield of *Leea aequata* L. Leaf extracts

Extraction Method	Yield (%)
Maceration	26.0
Reflux	11.0
UAE	20.0
MAE	29.5

The dried plant material met the quality standards of the Indonesian Herbal Pharmacopoeia, with moisture content and loss on drying below 10% (Table 1), indicating good stability and low susceptibility to microbial and enzymatic degradation (Dyah et al., 2025). The higher ethanol-soluble than water-soluble extractive value suggests that *L. aequata* leaves are dominated by semipolar-to-polar constituents such as flavonoids, tannins, and phenolics, consistent with other ethanol-extracted, phenolic-rich medicinal plants (Christian et al., 2025; Dyah et al., 2025).

Extraction Yield

Extraction yield is a key parameter for evaluating extraction efficiency, as different techniques can affect the quantity of phytochemicals recovered due to differences in mechanism, temperature, solvent penetration, and mass-transfer efficiency.

MAE produced the highest yield, followed by maceration, UAE, and reflux (Table 2), reflecting how extraction

Table 3. Phytochemical screening results of *Leea aequata* L. leaf extract

Secondary Metabolites	Maceration	Reflux	UAE	MAE
Phenolics	(+)	(+)	(+)	(+)
Flavonoids	(+)	(+)	(+)	(+)
Tannins	(+)	(+)	(+)	(+)
Saponins	(+)	(+)	(+)	(+)
Steroids/ Triterpenoids	(+) Triterpenoids	(+) Triterpenoids	(+) Triterpenoids	(+) Triterpenoids

Note : (+) indicates the presence of the compound class

temperature, solvent penetration, time, and cell-disruption mechanism affect metabolite recovery (Yuan et al., 2026). The superior yield of MAE likely stems from microwave-induced dielectric heating, which rapidly raises intracellular pressure, ruptures cell walls, and accelerates compound release while minimizing thermolabile degradation (Bhadange, Carpenter, & Saharan, 2024; Monton et al., 2022). Reflux gave the lowest yield, likely due to prolonged heat exposure degrading thermosensitive phenolics (Ling et al., 2022; Shi et al., 2022; Calderón-Oliver & Ponce-Alquicira, 2021). Maceration's relatively high yield reflects effective passive diffusion of polar compounds despite longer processing time and greater solvent use (Goti, 2023), while UAE's moderate yield reflects cavitation-enhanced solvent penetration and cell disruption (Sethi & Rathod, 2026), though still less efficient than MAE's energy transfer. Overall, MAE emerges as an efficient, green extraction technology that reduces time, solvent, and energy consumption for pharmaceutical applications (Panichayupakaranant, 2026).

Phytochemical Screening

Phytochemical screening was performed to identify the secondary metabolites present in the ethanol extracts of *Leea aequata* L. leaves.

All four extraction methods produced qualitatively similar phytochemical profiles, testing positive for phenolics, flavonoids, tannins, alkaloids, saponins, and triterpenoids. Phenolics and tannins were detected via blue-black coloration from ferric-ion–hydroxyl complexation, consistent with their radical-stabilizing hydrogen/electron-donating capacity (El Rayess, Nehme, Azzi-Achkouty, & Julien, 2024; Hassanpour & Doroudi, 2023). Flavonoids produced yellow coloration through lead–hydroxyl/carbonyl complexation, supporting their recognized role as radical scavengers and metal chelators (Hassanpour & Doroudi, 2023; Kalauni, Karki, Sharma, & Khanal, 2024; Tumilaar et al., 2024). Alkaloids were confirmed by characteristic precipitates with Mayer's, Dragendorff's, and Wagner's reagents, indicating chemical diversity relevant to broader pharmacological potential (Kumar et al., 2023; Shalihin,

Table 4. Linear regression parameters and IC_{50} values of reference antioxidants *Leea aequata* L. leaf extracts

Sample	Regression equation	R^2	IC_{50} value (ppm)	Antioxidant activity category
Vitamin C	y = 7.6843x + 36.4551	0.9942	5.828 ± 0.03[a]	Very strong
Quercetin	y = 9.7385x + 27.5899	0.9937	9.986 ± 0.04[b]	Very strong
MAE	y = 11.2473x + 21.5153	0.9939	12.586 ± 0.04[c]	Very strong
UAE	y = 15.6387x – 3.0783	0.9928	29.786 ± 0.05[d]	Very strong
Maceration	y = 16.4932x – 9.8454	0.9924	37.656 ± 0.06[e]	Very strong
Reflux	y = 15.8726x – 8.7810	0.9921	40.581 ± 0.07[f]	Very strong

Note : Different superscript letters in the same column indicate significant differences among samples based on one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Vitamin C ([a]), quercetin ([b]), MAE extract ([c]), UAE extract ([d]), maceration extract ([e]), and reflux extract ([f]) were significantly different from one another.

Muhaimin, & Latief, 2022). Saponins produced stable foam due to their amphiphilic structure and are linked to antioxidant, anti-inflammatory, antimicrobial, and cytoprotective activities (Shalihin et al., 2022; Timilsena, Phosanam, & Stockmann, 2023). The Liebermann–Burchard test yielded red-purple coloration, confirming triterpenoids rather than steroids, compounds associated with additional antioxidant and cytoprotective potential in Leea species (Purwaniati et al., 2024; Shalihin et al., 2022). Together, these metabolite classes provide the phytochemical basis for the antioxidant activity observed in the DPPH assay, consistent with prior reports on *L. aequata* (Hossain et al., 2023; Mostofa et al., 2024; Purwaniati et al., 2024).

Antioxidant Activity Using DPPH Assay

The IC_{50} (Table 4) values were lowest for vitamin C (5.828 ± 0.03 ppm) and quercetin (9.986 ± 0.04 ppm), followed among the plant extracts by MAE (12.586 ± 0.04 ppm), UAE (29.786 ± 0.05 ppm), maceration (37.656 ± 0.06 ppm), and reflux (40.581 ± 0.07 ppm), consistent with the DPPH hydrogen/electron-donation mechanism (Gulcin & Alwasel, 2023). High R^2 values (0.9921–0.9942) confirmed reliable regression models, and one-way ANOVA with Tukey's post hoc test showed significant differences among methods ($p < 0.05$, $p < 0.001$), confirming that extraction method, rather than random variation, drove the observed differences.

All extracts showed very strong antioxidant activity (IC_{50} below 50 ppm) (Indah, Lestari, Lasenda, & Sitindaon, 2025), with MAE closest to quercetin's activity. This superior performance likely reflects microwave-induced dielectric heating, which raises internal pressure, ruptures plant tissue, and accelerates release of DPPH-reactive compounds more effectively than maceration or reflux (Solaberrieta, Jiménez, & Garrigós, 2022; López-Salazar et al., 2023; Martins et al.,

2023). UAE's intermediate activity reflects cavitation-enhanced cell disruption and mass transfer (Legesse et al., 2024; Yusoff et al., 2022), while reflux's weakest activity likely results from thermal degradation of heat-sensitive phenolics during prolonged heating (Antony & Farid, 2022; Snoussi et al., 2022). These findings align with earlier reports of DPPH-scavenging activity in *L. aequata* leaves and fractions, although absolute IC_{50} values vary with extraction technique and assay conditions (Hossain et al., 2024; Mostofa et al., 2024).

Overall, extraction method markedly influenced antioxidant activity, with MAE identified as the most effective technique for maximizing the DPPH radical-scavenging activity of *L. aequata* leaves.

CONCLUSION

This study demonstrated that the extraction method significantly influenced the antioxidant activity of *Leea aequata* L. leaf extracts. Although maceration, reflux, ultrasound-assisted extraction, and microwave-assisted extraction produced similar qualitative phytochemical profiles, differences were observed in their extraction yields and DPPH radical-scavenging activities. Microwave-assisted extraction produced the highest yield (29.5%) and the highest antioxidant activity, as indicated by the lowest IC_{50} value of 12.586 ± 0.04 ppm, followed by ultrasound-assisted extraction (29.786 ± 0.05 ppm), maceration (37.656 ± 0.06 ppm), and reflux (40.581 ± 0.07 ppm). Therefore, microwave-assisted extraction was identified as the most effective method among the evaluated techniques for obtaining *L. aequata* leaf extract with the highest antioxidant activity. Further studies are recommended to quantify the total phenolic and flavonoid contents and identify the compounds responsible for the observed antioxidant activity.

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