Jurnal Jamu Indonesia (2026) 11(1): 52–57
https://jamu-journal.ipb.ac.id/index.php/JJI/index

ISSN 2407-7178
eISSN 2407-7763

Research Article
Doi: https://doi.org/10.29244/jji.v11i1.394

Comparative Analysis of Total Flavonoid Content and Antioxidant Activity in Medicinal Plant Leaf Extracts Using Ethanol and Citric Acid

Analisis Komparatif Kadar Flavonoid Total dan Aktivitas Antioksidan pada Ekstrak Daun Tanaman Obat Menggunakan Etanol dan Asam Sitrat

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ARTICLE INFO

Article history
Received on: 2025-04-25
Revised on: 2025-10-01
Accepted on: 2026-01-22

Keyword:
Antioxidants;
Citric Acid;
Ethanol;
Flavonoid;
Green Solvent

Kata kunci:
Antioksidan;
Asam Sitrat;
Etanol;
Flavonoid;
Pelarut Hijau

ABSTRACT

Flavonoids are bioactive compounds with antioxidant activity, and their extraction efficiency is strongly influenced by solvent selection. This study compared total flavonoid content and antioxidant activity of leaf extracts from *Persea americana*, *Annona muricata*, *Piper betle*, and *Pandanus amaryllifolius* obtained using ethanol and citric acid. Total flavonoid content was determined using the $AlCl_3$ spectrophotometric method with quercetin as the reference standard, while antioxidant activity was evaluated by the DPPH assay. Ethanol generally yielded higher flavonoid levels, with the highest content observed in *P. americana* leaves (3.87 mg QE/g), whereas citric acid extracts exhibited stronger antioxidant activity, reaching 94.21% DPPH inhibition in *P. betle* leaves. No significant correlation was observed between total flavonoid content and antioxidant activity. Overall, these results indicated that solvent selection critically determines extraction outcomes, and citric acid represents a viable green alternative for antioxidant-oriented applications.

ABSTRAK

Flavonoid merupakan senyawa bioaktif yang memiliki aktivitas antioksidan, dengan efisiensi ekstraksi yang sangat dipengaruhi oleh jenis pelarut. Penelitian ini membandingkan kadar flavonoid total dan aktivitas antioksidan ekstrak daun *Persea americana*, *Annona muricata*, *Piper betle*, dan *Pandanus amaryllifolius* menggunakan etanol dan asam sitrat. Kadar flavonoid total ditentukan dengan metode spektrofotometri $AlCl_3$ menggunakan kuersetin sebagai standar, sedangkan aktivitas antioksidan dianalisis dengan metode DPPH. Etanol umumnya menghasilkan kadar flavonoid lebih tinggi, dengan nilai tertinggi pada daun *P. americana* (3,87 mg QE/g), sedangkan ekstrak asam sitrat menunjukkan aktivitas antioksidan tertinggi pada daun *P. betle (94,21%)*. Tidak terdapat korelasi signifikan antara kadar flavonoid total dan aktivitas antioksidan. Secara keseluruhan, hasil ini menunjukkan bahwa pemilihan pelarut sangat menentukan hasil ekstraksi, dan asam sitrat berpotensi sebagai pelarut hijau alternatif untuk aplikasi antioksidan.

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Citation: Aziz, S., Mulyani, I., & Nadiah, S. (2026). Comparative Analysis of Total Flavonoid Content and Antioxidant Activity in Medicinal Plant Leaf Extracts Using Ethanol and Citric Acid. *Jurnal Jamu Indonesia*, *11*(1), 52–57. https://doi.org/10.29244/jji.v11i1.394

1. INTRODUCTION

Flavonoids are a major class of polyphenolic compounds widely distributed in plants and are known to exhibit diverse biological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer effects (Kumar et al., 2024; Vimalavathini et al., 2024). These biological properties have positioned flavonoids as promising candidates for the development of therapeutic agents targeting chronic diseases, cardiovascular disorders, and other degenerative conditions (Pattanaik et al., 2024; Qayum et al., 2024). The therapeutic effects of flavonoids are largely attributed to their ability to neutralize reactive oxygen species (ROS), induce apoptosis, and modulate key cellular signaling pathways such as mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), which play critical roles in inflammation and immune regulation (Ahmad et al., 2024; Chenchula et al., 2024).

In clinical and pharmaceutical applications, the efficacy of flavonoid-rich extracts has been strongly dependent on the concentration and purity of their bioactive constituents. Flavonoid content in plant materials has been reported to vary substantially depending on plant species, plant parts, and extraction methods, particularly the type of solvent employed (Lezoul et al., 2020). Therefore, the selection of an appropriate extraction method has been considered crucial to achieving optimal recovery of flavonoid compounds. Conventional extraction methods have commonly relied on organic solvents such as ethanol or methanol due to their effectiveness in solubilizing polar compounds; however, these solvents present limitations related to cost, residual toxicity, and environmental sustainability (Chemat et al., 2019).

With the growing emphasis on sustainable extraction practices, increasing efforts have been directed toward replacing conventional organic solvents with environmentally friendly alternatives, commonly referred to as green solvents. One promising alternative is citric acid solution, a weak organic acid naturally abundant in fruits. Citric acid has been reported to lower extraction pH, facilitate plant cell wall disruption, and form complexes with metal ions, thereby enhancing the release and stability of phenolic compounds, including flavonoids (Troter et al., 2016). Moreover, citric acid is biodegradable, readily available, and safer for both human health and the environment compared with conventional organic solvents.

Despite its potential advantages, comparative studies directly evaluating the effectiveness of citric acid solutions versus ethanol for flavonoid extraction remain limited. The use of 1% (w/v) citric acid in this study was based on previous findings demonstrating its effectiveness in enhancing phenolic and flavonoid extraction while minimizing the degradation of acid-sensitive compounds, making it suitable for green extraction systems (Melini et al., 2026). Therefore, generating comparative data on citric acid–based extraction is essential to support the development of efficient and sustainable extraction strategies with potential industrial applications. This study aimed to evaluate the effect of solvent type on total flavonoid content and antioxidant activity in leaf extracts of *Persea americana*, *Annona muricata*, *Piper betle*, and *Pandanus amaryllifolius*. Extraction was performed using the ultrasonic-assisted extraction (UAE) method with two solvents: 96% ethanol as a conventional solvent and 1% citric acid solution as an alternative green solvent. Additionally, the relationship between total flavonoid content and antioxidant activity was examined to assess the contribution of flavonoids to the overall bioactivity of the extracts.

2. METHODS

2.1. Plant Material Preparation

Fresh leaves of *P. americana*, *A. muricata*, *P. betle*, and *P. amaryllifolius* (500 g per species) were collected, manually sorted to remove damaged materials, washed thoroughly with running water, and air-dried. The plant materials were subsequently dried in a dehydrator (Wirastar, Semarang, Indonesia) at 40 ˚C for 48 h to minimize thermal degradation of heat-sensitive compounds. After drying, the samples were re-sorted and ground using a mechanical grinder to obtain homogeneous powdered crude drugs.

2.2. Determination of Moisture Content and Total Ash

Total ash content was determined by weighing 2.0 g of powdered sample into a pre-ignited silica crucible. The crucible was incinerated in a muffle furnace until a constant weight was achieved, and the ash content was calculated based on the dry weight of the sample (Kementerian Kesehatan RI, 2017).

Moisture content was determined using the azeotropic distillation method with toluene (Merck, Darmstadt, Germany). Approximately 20 g of powdered sample was distilled with 200 mL of water-saturated toluene, and the volume of condensed water was recorded to calculate moisture content (Kementerian Kesehatan RI, 2017).

2.3. Qualitative Identification of Flavonoids

Qualitative identification of flavonoids was conducted by heating 2 g of powdered sample with distilled water for 30 min, followed by cooling and filtration. The filtrate was treated with 5 N hydrochloric acid and magnesium powder, then homogenized and extracted with amyl alcohol. The formation of yellow, orange, or red coloration in the amyl alcohol layer indicated the presence of flavonoid compounds (Mulyani et al., 2021).

2.4. Ultrasonic-Assisted Extraction

For extraction, 2.0 g of powdered sample was transferred into an Erlenmeyer flask containing 25 mL of extraction solvent. Two solvent systems were employed: 96% ethanol (Brataco, Indonesia) as a conventional solvent and 1% (w/v) citric acid solution (Kimiamart, Surabaya, Indonesia) as an alternative green solvent. Extraction was carried out using ultrasonic-assisted extraction (UAE) (Elmasonic, Singen, Germany) for 30 min at room temperature to enhance solvent penetration and mass transfer. The

resulting extracts were filtered through Whatman No. 1 filter paper and directly subjected to further analyses.

2.5. Determination of Total Flavonoid Content

Total flavonoid content was determined using the aluminum chloride ($AlCl_3$) colorimetric method. Each extract was mixed sequentially with ethanol, 10% (w/v) $AlCl_3$ solution, 1 M sodium acetate, and distilled water, then incubated for 30 min at room temperature. Absorbance was measured at 415 nm using a UV–Vis spectrophotometer (Thermo Scientific, Massachusetts, USA) (Kementrian Kesehatan RI, 2017). Total flavonoid content was expressed as milligrams of quercetin equivalents per gram of sample (mg QE/g).

2.6. Determination of Antioxidant Activity

Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay following Faradilla et al., (2024), with minor modifications. Extract solutions (5 mg/mL) were mixed with DPPH solution (50 µg/mL) at a 1:1 (v/v) ratio and incubated for 30 min at room temperature in the dark. Ascorbic acid (Merck, Darmstadt, Germany) was used as a reference standard, and antioxidant activity was expressed as a percentage of DPPH radical inhibition.

2.7. Statistical Analysis

All experiments were performed in triplicate, and results were reported as mean ± standard deviation (SD). Data normality was assessed using the Shapiro–Wilk test. Differences among extraction solvents were analyzed using the Kruskal–Wallis test due to small sample size and non-normal data distribution. The association between total flavonoid content and antioxidant activity was evaluated using Spearman's rank correlation coefficient. All statistical analyses were conducted using SPSS software version 23.0 (IBM Corp., Armonk, NY, USA).

3. RESULTS AND DISCUSSION

3.1. Physicochemical Characterization of Crude Drugs

The yield of dried crude drugs varied among plant species, with *P. americana* leaves exhibiting the highest yield (38.46%) and *P. betle* leaves the lowest (9.30%). Such variation was primarily influenced by differences in moisture content and total ash levels. Lower moisture contents in *A. muricata* and *P. amaryllifolius* leaves (≈10%) resulted in higher crude drugs yields compared with *P. betle* leaves (11.91%), likely due to reduced mass loss during drying. Similar observations have been reported for medicinal plants with high moisture sensitivity during dehydration (Nurhaslina et al., 2022).

In addition, the relatively high total ash content of *P. americana* leaves (13.88%) reflected greater mineral retention, contributing to higher crude drugs yield. Conversely, the low yield of *P. betle* leaves was likely associated with the loss of volatile constituents during drying, a common phenomenon in aromatic plant materials (Nurhaslina et al., 2022). Overall, crude drugs yield was governed by the combined influence of moisture content, mineral composition, and volatility rather than by a single physicochemical parameter. The physicochemical characteristics of the crude drugs are summarized in **Table 1**.

Table 1. Physicochemical Characteristics of Dried Medicinal Plant Leaves (Crude Drugs).

Crude drugs (Leaves)	Crude drugs yield (%, w/w)	Moisture content (%, v/v)	Total ash content (%, w/w)
Persea americana	38.46	12.00	13.88
Annona muricata	36.05	10.00	6.77
Piper betle	9.30	11.91	10.00
Pandanus amaryllifolius	20.90	10.05	5.99

3.2. Total Flavonoid Content

Qualitative analysis confirmed the presence of flavonoid compounds in all crude drugs samples. Total flavonoid content (TFC), expressed as mg quercetin equivalents per gram of dry extract (mg QE/g), varied depending on both plant species and extraction solvent (**Figure 1**).

Extraction using 96% ethanol generally produced higher TFC values than citric acid for most samples, except *A. muricata*. The highest TFC was observed in the ethanolic extract of *P. americana* leaves (3.87 ± 0.04 mg QE/g), whereas citric acid extraction yielded substantially lower values (0.28 ± 0.07 mg QE/g). Similar solvent-dependent trends were observed for *P. betle* and *P. amaryllifolius*. Ethanol is known to be effective for extracting moderately polar flavonoid aglycones due to its broad polarity range and hydrogen-bonding capacity (Saidman et al., 2002; Cartika et al., 2024).

In contrast, *A. muricata* leaves extracted with 1% citric acid exhibited slightly higher TFC than those extracted with ethanol, although the difference was not statistically significant. This result suggests that citric acid may favor the extraction of more polar flavonoid derivatives in specific plant matrices, consistent with previous reports on organic acid–based green solvents (Kurtulbaş et al., 2022; Song et al., 2023). Statistical analysis confirmed that differences in TFC between solvents were not significant overall (Kruskal–Wallis, $p = 0.094$), indicating that flavonoid recovery was strongly influenced by the interaction between solvent properties and plant matrix composition rather than solvent polarity alone.

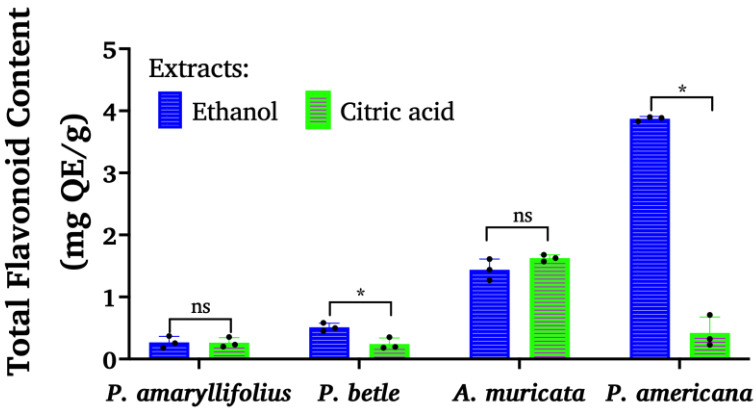

Figure 1. Total flavonoid content of medicinal plant leaf extracts obtained using ethanol and citric acid. Values are expressed as mg quercetin equivalents per gram of dry extract (mg QE/g). Error bars represent standard deviation (SD, n = 3). Asterisks (*) indicate statistically significant differences between solvents ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$).

3.3. Antioxidant Activity

All extracts demonstrated the ability to scavenge DPPH radicals, indicating inherent antioxidant potential (**Figure 2**). Antioxidant activity differed significantly between extraction solvents (Kruskal–Wallis, $p < 0.05$). In general, citric acid extracts exhibited higher antioxidant activity than ethanolic extracts, except for *P. amaryllifolius*.

The highest antioxidant activity was observed in *P. betle* leaves extracted with citric acid (94.21 ± 0.15%), which was markedly higher than that of the corresponding ethanolic extract (30.91 ± 0.15%). This finding was notable because ethanol extraction often yielded higher flavonoid contents, whereas citric acid extracts exhibited superior antioxidant activity in several samples. This discrepancy suggests that antioxidant capacity was not solely governed by total flavonoid concentration.

Phenolic compounds, including flavonoids, contribute to antioxidant activity through electron or hydrogen donation; however, organic acids such as citric acid can enhance antioxidant performance indirectly by stabilizing phenolic compounds and chelating pro-oxidant metal ions (Jeszka-Skowron et al., 2015; Zeb, 2020).

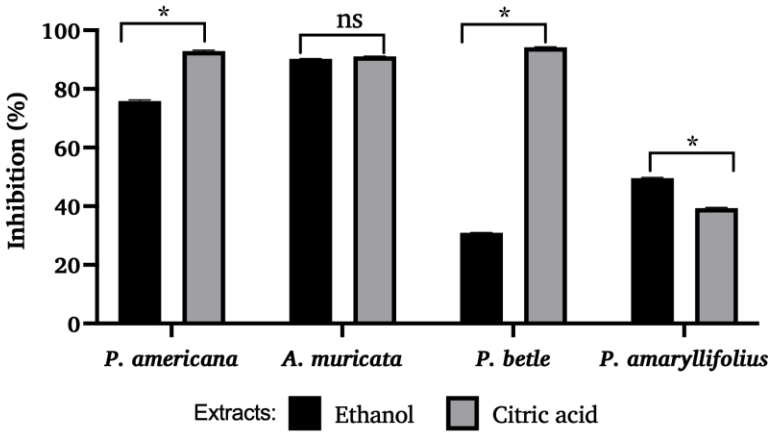

Figure 2. Antioxidant activity of medicinal plant leaf extracts determined by the DPPH radical scavenging assay. Values are expressed as percentage of DPPH inhibition. Error bars represent standard deviation (SD, n = 3). Asterisks (*) indicate a statistically significant difference between solvents ($p < 0.05$), while ns indicates no significant difference ($p > 0.05$).

3.4. Relationship Between Total Flavonoid Content and Antioxidant Activity

Spearman correlation analysis revealed a moderate positive correlation between TFC and antioxidant activity for ethanolic extracts ($\rho = 0.580$), although it was not statistically significant ($p = 0.480$). In contrast, citric acid extracts showed a weak and non-significant negative correlation ($\rho = -0.385$; $p = 0.217$) (**Table 2**). Similar inconsistencies between flavonoid content and antioxidant activity have been reported in various plant systems (Ramli, 2006; Khiya et al., 2021). These findings indicate that antioxidant activity could not be attributed to flavonoid content alone and likely reflects the combined contribution of multiple bioactive constituents, including phenolic acids, tannins, terpenoids, and non-phenolic metabolites (Hilal et al., 2024).

Table 2. Correlation between total flavonoid content and antioxidant activity of leaf extracts

Correlation parameter	Ethanol extract	Citric acid extract
Total flavonoid content vs. antioxidant activity	$\rho = 0.580$ ($p = 0.480$)	$\rho = -0.385$ ($p = 0.217$)

ρ indicates Spearman's rank correlation coefficient.

3.5. Solvent Effects and Extraction Implications

The present results demonstrate that solvent selection influenced not only flavonoid recovery but also the biological activity of the extracts. Ethanol was generally more effective for extracting flavonoids from *P. americana*, *P. betle*, and *P. amaryllifolius*, in agreement with previous studies on flavonoid-rich plant materials (Mulyono et al., 2022; Trifunschi & Ardelean, 2013). Conversely, citric acid showed better performance in *A. muricata*, likely due to its high polarity and ability to interact with polar phenolic compounds.

As a weak organic acid, citric acid undergoes stepwise dissociation in aqueous solution, generating citrate species capable of hydrogen bonding and electrostatic interactions with phenolics, thereby enhancing solubility and stability (Kurtulbaş et al., 2022; Song et al., 2023). Combined with ultrasonic-assisted extraction, which promotes cell wall disruption and mass transfer, citric acid represents a promising green solvent for antioxidant-oriented extraction (Tušek et al., 2022).

4. CONCLUSION

This study demonstrated that solvent type significantly affected the total flavonoid content and antioxidant activity of leaf extracts from *P. americana*, *A. muricata*, *P. betle*, and *P. amaryllifolius*. Extraction using 96% ethanol generally produced higher total flavonoid content, with the highest value observed in *P. americana* leaves (3.87 mg QE/g). In contrast, extraction with 1% citric acid resulted in higher antioxidant activity in most samples, reaching 94.21% DPPH radical inhibition in *P. betle* leaves. No significant correlation was observed between total flavonoid content and antioxidant activity. Further studies are recommended to characterize the specific bioactive compounds contributing to antioxidant activity using advanced phytochemical profiling techniques.

AUTHOR CONTRIBUTIONS

Conceptualization, SA and IM; methodology, SA and IM; software, SA; validation, SA, IM, and SN; formal analysis, SA; investigation, SA and SN; resources, IM; data curation, SA; writing—original draft preparation, SA and SN; writing—review and editing, IM; visualization, SN; supervision, SA; funding acquisition, not applicable. All authors have read and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable.

INFORMED CONSENT STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

FUNDING

This research received no external funding.

ACKNOWLEDGMENT

The authors gratefully acknowledge Faculty of Science, Institut Teknologi Sumatera (Itera) for providing research facilities and laboratory support.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) USE

During the preparation of this manuscript, the author(s) used ChatGPT (OpenAI) and Consensus to assist in improving the clarity, structure, or readability of the text. After using this tool, the author(s) thoroughly reviewed, edited, and verified the entire content to ensure it accurately represents their own ideas and interpretations. The author(s) take full responsibility for the integrity and originality of the published work.

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