
COMPARISON OF EXTRACTION METHODS ON ANTIOXIDANT ACTIVITY, PHENOLIC, FLAVONOIDS, AND TANNINS TOTAL OF METHANOL EXTRACTS FROM KAWISTA LEAVES (*Limonia Acidissima* L.)

Amanda Wahyu Sabrina, Anita Dwi Puspitasari*, Sugito Chandra

Fakultas Farmasi, Universitas Wahid Hasyim

Jl. Raya Gunungpati Kilometer 15, Nongkosawit, Semarang 50224

*Email: anita@unwahas.ac.id

Abstract

Antioxidants are compounds that can counteract free radicals and protect the body from degenerative diseases, such as heart disease, cancer, and diabetes. Kawista leaves (*Limonia acidissima* L.) are a natural source of antioxidants containing secondary metabolites, namely phenols, flavonoids, and tannins. Therefore, selecting the appropriate extraction method is crucial for optimizing the yield of secondary metabolites and their biological activity. This study aims to compare extraction methods (maceration, percolation, reflux, and soxhlet) in terms of antioxidant activity, total phenolic, flavonoid, and tannin content from kawista leaf methanol extracts. Antioxidant activity was analyzed using the DPPH method, while total phenolic, flavonoid, and tannin content were determined using the Folin-Ciocalteu, $AlCl_3$, and Folin-Denis methods, respectively. The results showed that the percolation method yielded the highest extract yield (23.3%) compared to reflux (18.9%), soxhlet (13.9%), and maceration (11%). The Soxhlet extract showed superior antioxidant activity, as indicated by a lower IC_{50} (93.21 ppm) compared to the maceration (119.81 ppm), reflux (114.01 ppm), and percolation (128.71 ppm) methods. The highest total levels of phenolic, flavonoid, and tannin were also observed in the soxhlet extract, with concentrations of 92.48 mg GAE/g, 6.49 mg QE/g, and 97.85 mg TAE/g, respectively. Thus, differences in extraction methods can affect the antioxidant activity and levels of secondary metabolites in methanol extracts of kawista leaves.

Keywords: Antioxidants, Kawista Leaves, DPPH, Extraction Methods

Abstrak

Antioksidan merupakan senyawa yang dapat menangkal radikal bebas dan melindungi tubuh dari penyakit degeneratif, seperti penyakit jantung, kanker, dan diabetes. Daun kawista (*Limonia acidissima* L.) merupakan salah satu sumber antioksidan alami yang mengandung senyawa metabolit sekunder, yaitu fenol, flavonoid, dan tanin. Oleh karena itu, pemilihan metode ekstraksi yang tepat sangat penting untuk mengoptimalkan perolehan kadar metabolit sekunder serta aktivitas biologisnya. Penelitian ini bertujuan untuk membandingkan metode ekstraksi (maserasi, perkolasi, refluks, dan soxhlet) terhadap aktivitas antioksidan, kadar fenolik, flavonoid, dan tanin total dari ekstrak metanol daun kawista. Analisis aktivitas antioksidan dilakukan dengan metode DPPH, sedangkan penetapan kadar fenolik, flavonoid, dan tanin masing-masing menggunakan metode Folin-Ciocalteu, $AlCl_3$, dan Folin-Denis. Hasil penelitian menunjukkan bahwa metode perkolasi memberikan rendemen ekstrak yang paling besar, yaitu sebesar 23,3% dibandingkan dengan refluks (18,9%), soxhlet (13,9%), dan maserasi (11%). Ekstrak hasil soxhlet menunjukkan aktivitas antioksidan yang lebih tinggi ditandai dengan nilai IC_{50} yang lebih kecil (93,21 ppm) dibanding metode maserasi (119,81 ppm), refluks (114,01 ppm), dan perkolasi (128,71 ppm). Kadar fenolik, flavonoid, dan tanin total tertinggi juga diperoleh pada ekstrak hasil soxhlet berturut-turut 92,48 mg GAE/g, 6,49 mg QE/g, dan 97,85 mg TAE/g. Dengan demikian, perbedaan metode ekstraksi dapat mempengaruhi aktivitas antioksidan dan kadar senyawa metabolit sekunder pada ekstrak metanol daun kawista.

Kata kunci: Antioksidan, Daun Kawista, DPPH, Metode Ekstraksi

INTRODUCTION

Excessive free radicals can lead to chronic diseases such as heart attacks, cancer, and diabetes. Free radicals are molecules that lose electrons from their free electron pairs, making them unstable and prone to reacting with other molecules, forming new radicals (Chandimali *et al.*, 2025). Free radicals are chemically defined as molecules that have lost one electron from their pair of free

electrons. As a result, they are extremely reactive and frequently attack other molecules to create new radicals (Martemucci *et al.*, 2022). A compound that can donate electrons (electron donor) and inhibit oxidation reactions by binding free radicals and reactive molecules without becoming a free radical itself is called an antioxidant (Gulcin, 2025). There are two types of antioxidants: endogenous and exogenous antioxidants. The body is capable of producing endogenous antioxidants, but if free radicals are excessive, the body requires external antioxidant assistance. Antioxidants that come from outside the body are of two types: natural and synthetic. Synthetic antioxidants come from chemical reaction synthesis, which tend to have adverse effects on the body, whereas natural antioxidants come from plants that contain secondary metabolite compounds, making natural antioxidants safer to use (Hassanpour dan Doroudi, 2023).

One of the natural materials with potential as a natural antioxidant is the kawista leaf (*Limonia acidissima* L.). Kawista leaves are known to contain various secondary metabolites such as phenolics, flavonoids, tannins, saponins, and terpenoids (Ridwanuloh & Mursal, 2018). These compounds play an important role in capturing free radicals and providing other health benefits. Previous research reported that methanol extracts of kawista leaves showed antioxidant activity with an IC₅₀ value of 152 µg/mL (Attarde *et al.*, 2011). Azizah *et al.* (2024) also reported that methanol, 70% ethanol, and 96% ethanol extracts of kawista leaves obtained through maceration had IC₅₀ values of 82.20, 135.50, and 123.59 ppm, respectively, using the DPPH method, thereby strengthening the evidence that kawista leaves contain potential natural antioxidants.

Extraction methods are an important factor that can influence the yield, secondary metabolite content, and biological activity of an extract (Yudistirani & Islam, 2019). Some commonly used extraction methods include maceration, percolation, Soxhlet, and reflux (Mukhrani, 2014). Each method has a different working principle. Maceration is a simple soaking method without heating; percolation uses a flowing solvent system, Soxhlet utilizes continuous circulation of hot solvent, while reflux involves solvent circulation with heating for a specific period (Nugroho, 2017).

The ethanolic extract of *Limonia acidissima* leaves, obtained via maceration, exhibits strong antioxidant activity with DPPH IC₅₀ and CUPRAC values of 10.445 µg/mL and 135.42 µg/mL, respectively, and contains 14.63 mg GAE/g of total phenolics and 113.9 mg QE/g of total flavonoids (Fawwas *et al.*, 2024). Differences in extraction methods have been shown to produce varying levels of bioactive compounds. Verawati *et al.* (2017) reported that in salam leaves (*Syzygium polyanthum*), the percolation method produced the highest yield and phenolic content compared to the maceration, soxhlet, and reflux methods, but the best antioxidant activity was obtained from the maceration method. Safitri *et al.* (2018) showed that the percolation method produced the highest total phenolic and flavonoid content in beluntas leaves (*Pluchea indica* L.), while Nurhasnawati *et al.* (2017) found that the ethanol extract of jambu bol leaves (*Syzygium malaccense* L.) using the soxhlet method had better antioxidant activity compared to maceration.

Based on a review of existing literature, the extraction of *Limonia acidissima* leaves has only been reported using a single extraction method (Azizah *et al.*, 2024; Fawwas *et al.*, 2024; Febriana *et al.*, 2024). Research on the comparison of extraction methods for kawista leaves has not been widely reported, especially those that examine antioxidant activity as well as total phenolic, flavonoid, and tannin content simultaneously. Therefore, this study was conducted to compare the effects of various extraction methods, namely maceration, percolation, reflux, and Soxhlet, on the antioxidant activity, total phenolic, flavonoid, and tannin content of methanol extracts of kawista leaves (*Limonia acidissima* L.).

METHODOLOGY

Materials and Tools

The material used was kawista leaves (*Limonia acidissima* L.) collected from Desa Landoh, Suilang Subdistrict, Rembang Regency, Central Java. Harvesting took place in the morning between 08:00 and 10:00 WIB; the plants were approximately five years old, and the leaves selected were mature, dark green, and free of yellowing. Methanol (Merck) was used as the solvent for extraction, along with technical ethanol, distilled water, quercetin (Sigma), 10% AlCl₃ solution (Merck), FeCl₃, concentrated hydrochloric acid (HCl) concentrate, magnesium powder (Mg), NaCl, gelatin, potassium acetate, Folin-Ciocalteu reagent, gallic acid, tannic acid, vitamin C, Na₂CO₃, and DPPH (1,1-diphenyl-2-picrylhydrazine).

The equipment used includes a maceration vessel, a percolator, a siphon, a reflux condenser, a separating funnel, a round-bottom flask, a moisture balance (Ohaus), test tubes, measuring cups, beakers, porcelain dishes, a vacuum pump, pipettes, metal spoons, spatulas, stirring rods, watch glasses, filter paper (blue tip, yellow tip), UV-Vis Spectrophotometer -Vis 1800 (Shimadzu), electronic balance, analytical balance, rotary vacuum evaporator (Heidolph), and micropipettes.

Production of Kawista Leaf Herb Powder

A total of 5 kg of kawista leaves were first sorted to remove impurities, then washed under running water and drained. The kawista leaves were placed individually on a baking sheet and dried in an oven at 50°C until the moisture content fell below 10%. The moisture content was measured using a moisture balance.

Extraction Methods

a. Maceration

A maceration jar was filled with 100 g of kawista leaf powder and 750 mL of methanol solvent. For three days, the maceration procedure was conducted with twice-daily stirring. The mixture was filtered after three days to produce macerate (1). After being treated with an extra 250 mL of methanol, the residue was macerated once more for two days before being filtered to produce macerate (2). After combining macerates (1) and (2) and letting them settle overnight, they were separated from the residue and concentrated at 60°C in a rotary vacuum evaporator to produce a thick extract.

b. Soxhlet

One hundred grams of dried kawista leaves were enveloped in filter paper, secured at both ends, and placed into the extraction apparatus. Methanol (600 mL) was introduced into the system to initiate two circulation cycles, and boiling chips were added to the round-bottom flask to ensure even boiling. Following the attachment of a reflux condenser, the extraction was conducted over a water bath at 50°C. The process was maintained until the solvent in the siphon tube became colorless, indicating exhaustive extraction. The resulting liquid extract was subsequently concentrated using a rotary vacuum evaporator at 60°C to yield a viscous crude extract.

c. Percolation

Place 100 grams of dried kawista leaves into a beaker, add 200 mL of methanol to moisten the leaves, stir until evenly mixed, and then let it sit for a while. The moistened crude powder is placed in a percolator equipped with filter paper and porous cotton/sponge, then the solvent is added in small amounts until the liquid begins to drip, ensuring a layer of solvent remains above the crude drug (1–2 cm above the sample surface). Let stand for 24 hours at room temperature under covered conditions. The rate of solvent drainage is adjusted, and solvent is added continuously. The process is repeated several times until the solvent exiting the apparatus is colorless or clear. The percolate is collected in a beaker and concentrated using a rotary vacuum evaporator at a temperature of 60°C until a thick extract is obtained.

d. Reflux

One hundred grams of dried Kawista leaves were placed in a round-bottom flask, then 300 mL of methanol solvent was added. The reflux extraction process was carried out three times, each for 2 hours. After the reflux process was complete, the solution was filtered and then concentrated using a rotary vacuum evaporator at 60°C until a viscous extract was obtained.

The yield of the kawista leaf methanol extract was calculated using the following formula:

$$\text{Yield of the extract} = \frac{\text{Weight of concentrate extract (gram)}}{\text{weight of crude powder (gram)}} \times 100 \%$$

Phytochemical Screening

a. Flavonoid

One gram of kawista leaf methanol extract was dissolved in analytical-grade ethanol. An additional 5 mL of ethanol, a sufficient amount of magnesium reagent, and 5 drops of concentrated hydrochloric acid (HCl) were then introduced to the solution. After allowing the mixture to cool, it was shaken to observe any color variations. The emergence of an orange, light red, red, or purple ring confirms a positive reaction of flavonoid (Mufidah *et al.*, 2025).

b. Phenolic

One gram of kawista leaf methanol extract was weighed and dissolved in 5 mL of pro-analysis (p.a.) ethanol. Subsequently, three drops of 1% FeCl₃ reagent were added; the mixture was then shaken, and any color change was observed. A positive reaction is indicated by a color change to greenish-black (Rahimah *et al.*, 2019).

c. Tannins

One gram of kawista leaf methanol extract was weighed and dissolved in 5 mL of analytical-grade (p.a.) ethanol; subsequently, 5 mL of 10% NaCl solution was added, followed by the addition of 2–3 drops of gelatin solution. The mixture was stirred gently. A positive reaction was indicated by the formation of a white precipitate (Rauif & Saranani, 2023).

Determination of Total Phenolic, Flavonoid, and Tannin Content

Total phenolic content was determined using the Folin Ciocalteu method, in which phenolic compounds in the extract react with the Folin Ciocalteu reagent and sodium carbonate to form a blue color, the intensity of which is measured at a wavelength of 765 nm. Total flavonoid content was analyzed using the AlCl₃ complexation method, yielding a yellow-colored complex measured at a wavelength of 415 nm. Meanwhile, total tannin content was determined using the Folin Denis method, producing a dark blue complex measured at a wavelength of 725 nm (Pérez *et al.*, 2023).

Quantification of the three compounds was performed by comparing sample absorbance against standard curves: gallic acid for phenolics, quercetin for flavonoids, and tannic acid for tannins. Measurement results were expressed as mg of standard equivalent per gram of extract (mg GAE/g, mg QE/g, and mg TAE/g). This method enabled the accurate determination of phenolic, flavonoid, and tannin levels in the methanolic extract of kawista leaves, thereby supporting the analysis of the relationship between secondary metabolites and antioxidant activity.

Antioxidant Activity

The antioxidant activity of the methanolic extract of kawista leaves was evaluated using the DPPH free radical scavenging method. The purple DPPH solution turns pale yellow upon interaction with antioxidant compounds. Extract samples were prepared at various concentrations, reacted with the DPPH solution, and incubated for 30 minutes in the dark at room temperature. Absorbance was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer, and the percentage of inhibition was calculated by comparing the sample absorbance to that of the control. The IC₅₀ value was determined via linear regression between extract concentration and percentage inhibition, where IC₅₀ represents the concentration capable of inhibiting 50% of free radicals. The antioxidant activity of the methanol extract of kawista leaves was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method, in which the purple DPPH solution turns pale yellow upon interaction with antioxidant compounds. A 0.1 mM DPPH solution was prepared in methanol and stored in the dark to prevent photodegradation. The kawista leaf extract was dissolved in methanol to prepare a stock solution, from which serial dilutions were made to obtain at least five different concentrations (10, 20, 40, 80, and 160 µg/mL). For the assay, 2 mL of DPPH solution was mixed with 2 mL of each extract concentration in test tubes or microplate wells, while the control consisted of DPPH solution mixed with methanol alone. The mixtures were vortexed briefly to ensure homogeneity and then incubated in the dark at room temperature (20–25 °C) for 30 minutes to allow the reaction to proceed. After incubation, the absorbance of each solution was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer. The IC₅₀ value, representing the concentration of extract required to inhibit 50% of DPPH radicals, was determined by plotting extract concentration (µg/mL) on the x-axis against percentage inhibition on the y-axis and performing linear regression analysis; the IC₅₀ was then calculated from the regression equation by solving for the concentration at which inhibition equals 50%.

Data Analysis

Data were analyzed by expressing the measurement results as the mean ± standard deviation (SD) based on 3 independent replicates for each extraction method. The normality of the data was assessed using the Shapiro Wilk test, while homogeneity of variance was evaluated using Levene's test. When the assumptions of normality and homogeneity were satisfied, differences among

extraction methods were analyzed using one-way analysis of variance (ANOVA) at a 95% confidence level ($\alpha=0.05$). If the ANOVA showed a significant difference, Tukey's honestly significant difference (HSD) post-hoc test was performed to identify the specific pairs of groups that differed significantly. When the assumptions for parametric analysis were not met, the Kruskal Wallis test was used as a non-parametric alternative. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Results of Kawista Leaf Powder Production

A total of 5 kg of fresh Kawista leaves was used for this study. Upon drying, the leaves weighed 1.950 kg. The dried leaves were then pulverized, yielding 1.950 kg of powder. Moisture content analysis of the Kawista leaf powder showed it met the quality standard of 7%; generally, the moisture content requirement for crude plant material is no more than 10% (Depkes RI, 1985).

Extraction Results and Yield

The extraction process yielded a filtrate, which was subsequently processed using a rotary evaporator. The rotary evaporator operates on the principle of solvent evaporation, leaving behind only the compounds constituting the extract (Reo *et al.*, 2017). Extract yield refers to the amount of extract obtained from the extraction of the raw plant material, expressed as a percentage of the dry weight. Calculating the extract yield serves to determine the efficiency of the extraction process and to compare the efficacy of different solvents in extracting secondary metabolites. The extract yields obtained from the four extraction methods are presented in Table 1.

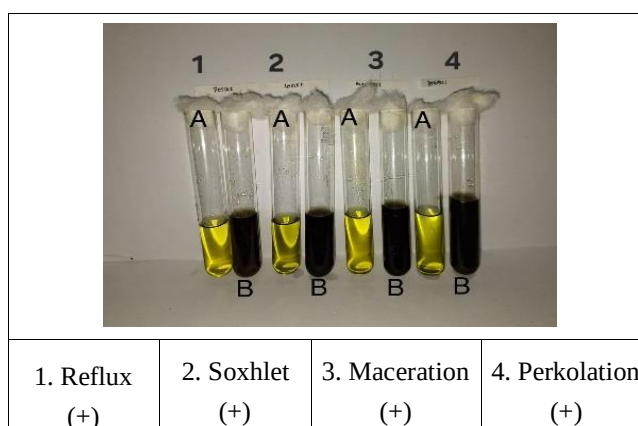
Table 1. The yield of Kawista leaf extract

Extraction methods	Weight of crude powder (g)	Weight of extract (gram)	Yield (%)
Maceration	100	11	11
Percolation	100	23,3	23,3
Reflux	100	18,9	18,9
Soxhlet	100	13,9	13,9

Phytochemical Screening Results

a. Phenolic

Phenolic testing was conducted using FeCl_3 . A positive result indicating the presence of phenolic compounds is marked by the formation of a blue, blue-green, or blue-black color upon reaction with FeCl_3 . The formation of a blue-black color is attributed to the reaction between Fe^{3+} and the keto groups present in the phenolic compounds (Syamsudin *et al.*, 2022). The results of the phenolic content identification are shown in Figure 1.

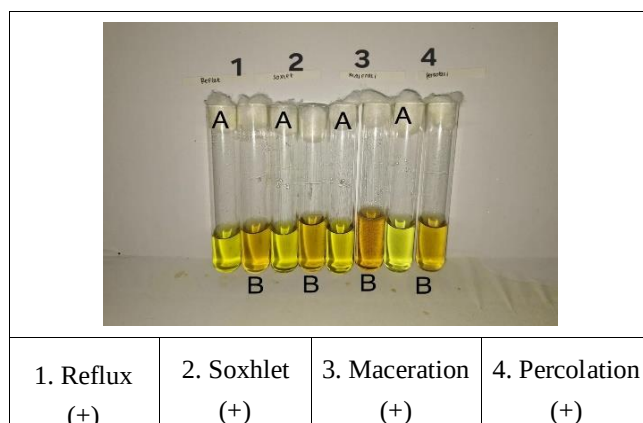


Note: A = Kawista leaf extract sample blank solution
B = Kawista leaf extract sample test solution + reagent

Figure 1. Phenolic Phytochemical Screening Results

b. Flavonoid

The methanol extract of kawista leaves was screened for flavonoids using concentrated HCl and a small amount of Mg powder. A sample was considered positive if an orange color developed; this occurs because Mg ions reduce the carbonyl group, yielding flavylum (Syamsudin *et al.*, 2022). The results of the flavonoid identification are shown in Figure 2.

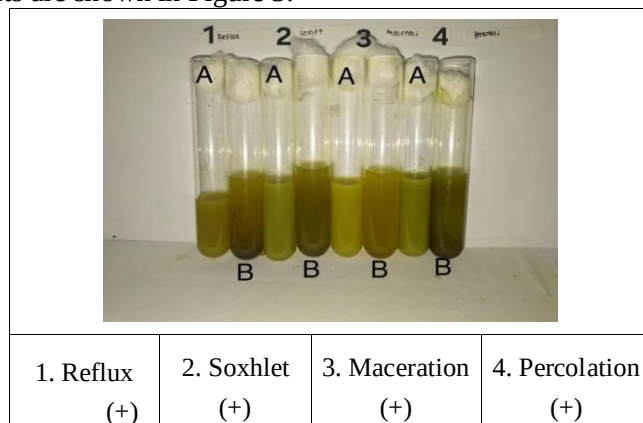


Note: A = Kawista leaf extract sample blank solution
B = Kawista leaf extract sample test solution + reagent

Figure 2. Flavonoid Phytochemical Screening Results

c. Tannins

Phytochemical screening was followed by the identification of tannins in the methanol extract of kawista leaves. Tannin identification was performed using 10% NaCl and a few drops of gelatin. Tannins are capable of binding to proteins, including gelatin, to form a precipitate. A positive result was indicated by the formation of a white or cloudy precipitate in the test tube. The identification results are shown in Figure 3.



Note: A = Kawista leaf extract sample blank solution
B = Kawista leaf extract sample test solution + reagent

Figure 3. Tannins Phytochemical Screening Results

Results of Total Phenolic, Flavonoid, and Tannin Content Determination

a. Phenolic

The determination of total phenolic content in the methanol extract of kawista leaves was performed using the Folin–Ciocalteu method, employing gallic acid as a standard alongside Folin–Ciocalteu reagent and sodium carbonate. The principle of this method involves the reduction of a phosphomolybdate-phosphotungstate mixture by the aromatic groups of phenolic compounds under alkaline conditions, resulting in the formation of a blue-colored complex. The intensity of the resulting color is proportional to the amount of phenolate ions formed; the higher

the phenolic content in the sample, the more intense the blue color (Kalpoutzakakis *et al.*, 2023). The results of the phenolic content determination are presented in Table 2.

Table 2. Total Phenolic Content of Methanol Extract of Kawista Leaves

Extraction methods	Average Total Phenolic Content (mgGAE/g extract) $\pm$ SD
Maceration	83.06 $\pm$ 0.29
Percolation	91.28 $\pm$ 0.58
Reflux	62.83 $\pm$ 1.28
Soxhlet	92.48 $\pm$ 0.86

The methanol extract of kawista leaves exhibited varying phenolic contents depending on the extraction method used. The highest value was obtained using the Soxhlet method (92.48 mg GAE/g extract), followed by the reflux, maceration, and percolation methods. Statistical analysis (ANOVA) revealed significant differences ($p < 0.05$) in phenolic content among the methods. A Tukey post-hoc test showed that the phenolic content obtained via the Soxhlet method differed significantly from that of the maceration, percolation, and reflux methods. These results indicate that the repeated heating process inherent to the Soxhlet method enhances the solubility and release of phenolic compounds, resulting in a higher phenolic yield. The combination of temperature, continuous solvent flow, and prolonged extraction time makes the Soxhlet method superior for extracting phenolic compounds compared to maceration, percolation, and reflux methods (Cao *et al.*, 2025).

b. Flavonoid

Total flavonoid content was determined using the $AlCl_3$ complexation method, with quercetin as the reference standard. The principle of this method involves the formation of a complex between the flavonoid hydroxyl groups and $AlCl_3$, resulting in a yellow color. The intensity of the color produced is proportional to the flavonoid concentration in the sample; thus, a higher flavonoid content results in a more intense color. The results of the flavonoid content determination are presented in Table 3.

Table 3. Total Flavonoid Content of Methanol Extract of Kawista Leaves

Extraction methods	Average Total Flavonoid Content (mgQE/g extract) $\pm$ SD
Maceration	4.09 $\pm$ 0.16
Percolation	3.43 $\pm$ 0.02
Reflux	3.14 $\pm$ 0.09
Soxhlet	6.49 $\pm$ 0.20

The determination of total flavonoid content showed the highest value with the Soxhlet method (6.49 mg QE/g extract), whereas the other methods yielded lower levels. ANOVA revealed a significant difference ($p < 0.05$), and the Tukey test indicated that the flavonoid content obtained via the Soxhlet method differed significantly from that of maceration and percolation but did not differ significantly from that of reflux. This suggests that the heating involved in both Soxhlet and reflux methods facilitates flavonoid extraction; however, the Soxhlet method yields more optimal results due to the continuous circulation of the solvent (Isnindar & Luliana, 2025).

Although the percolation method produced the highest recovery (yield) of crude extract, it resulted in a relatively low total phenolic content per gram of extract. This discrepancy arises because recovery reflects the total mass of all soluble compounds extracted, whereas total phenolic content expresses the concentration of phenolics within that extract. Percolation, which operates at ambient or mild temperatures with continuous solvent flow, efficiently removes a broad range of polar constituents (e.g., sugars, proteins, and other non-phenolic metabolites), thereby increasing the overall extract mass but diluting the phenolic fraction. In contrast, heated methods such as Soxhlet and reflux enhance the disruption of plant cell matrices and improve the solubility and release of phenolic compounds, leading to extracts that are richer in phenolics per unit mass even if the total crude yield is lower.

c. Tannins

Total tannin content was analyzed using the Folin–Denis method, with tannic acid serving as the reference standard. In this method, tannin compounds react with the Folin–Denis reagent to form a dark blue complex. The intensity of the resulting color is proportional to the amount of tannin in the sample; thus, a higher tannin content results in a more intense blue color. The results of the tannin content determination are presented in Table 4.

Table 4. Total Tannins Content of Methanol Extract of Kawista Leaves

Extraction methods	Average Total Tannins Content (mgTAE/g extract) $\pm$ SD
Maceration	62.82 $\pm$ 0.22
Percolation	56.72 $\pm$ 1.91
Reflux	38.34 $\pm$ 0.96
Soxhlet	97.85 $\pm$ 2.12

Analysis of total tannin content revealed the highest value in the Soxhlet extract (97.85 mg TAE/g extract), whereas the maceration, percolation, and reflux methods yielded lower levels. Statistical analysis (ANOVA) confirmed a significant difference ($p < 0.05$) among the methods. Tukey's post-hoc test results indicated that the Soxhlet method differed significantly from the other three methods. This reinforces the evidence that the Soxhlet method, involving continuous heating, is more effective at solubilizing polar tannin compounds than methods lacking intensive heating (Castillo-Correa *et al.*, 2025).

Antioxidant Activity

The antioxidant activity of the methanol extract of kawista leaves was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. DPPH is a stable, dark purple free radical compound widely used as a standard reagent in antioxidant activity assays due to its convenience, speed, and sensitivity. The principle of this method relies on the ability of antioxidant compounds within the extract to donate hydrogen atoms or electrons, thereby neutralizing DPPH free radicals. This reaction causes the DPPH solution to change color from purple to pale yellow, a change subsequently measured using a UV-Vis spectrophotometer at a wavelength of 517 nm. We used vitamin C as a standard. Vitamin C is used in the DPPH antioxidant assay as a standard or positive control because it is a well-known antioxidant with strong electron- and hydrogen-donating abilities. It reacts with the stable DPPH free radical by donating an electron or hydrogen atom, reducing the purple-colored DPPH radical to the pale-yellow non-radical form, DPPH-H. This reaction decreases the absorbance, usually measured at approximately 517 nm. The antioxidant activity of the test sample can then be compared with that of vitamin C. Therefore, vitamin C helps confirm that the assay is working properly and provides a reference for evaluating the radical-scavenging activity of the tested extract or compound.

During testing, kawista leaf extracts were prepared at various concentrations, reacted with a DPPH solution, and incubated for 30 minutes in the dark at room temperature to ensure an optimal reaction. The percentage of inhibition was calculated by comparing the sample's absorbance value to that of the control; thus, a higher percentage of inhibition indicates a stronger capacity of the extract to scavenge free radicals. The measurement results were subsequently processed using linear regression analysis, plotting extract concentration against the percentage of inhibition, to determine the IC_{50} value. The IC_{50} (Inhibitory Concentration 50) value represents the extract concentration capable of scavenging 50% of DPPH free radicals. A lower IC_{50} value indicates higher antioxidant activity. Thus, the DPPH method can be used to assess the antioxidant potency of kawista leaf extracts and to compare the effectiveness of different extraction methods. The antioxidant activity of the methanolic kawista leaf extract is presented in Table 5.

Table 5. Determination of Antioxidant Activity of Kawista Leaf Methanol Extract

Extraction methods	Average value of IC ₅₀ ± SD	Note
Maceration	119.81 ± 0.44	Moderate
Percolation	128.71 ± 0.44	Moderate
Reflux	114.01 ± 0.41	Moderate
Soxhlet	93.21 ± 0.45	Strong

The antioxidant activity of the extracts was evaluated using the DPPH method. The results indicated that the Soxhlet method yielded the lowest IC₅₀ value (93.21 ppm), followed by reflux (114.01 ppm), maceration (119.81 ppm), and percolation (128.71 ppm). A lower IC₅₀ value indicates stronger antioxidant activity. Statistical analysis (ANOVA) revealed significant differences ($p < 0.05$) among the extraction methods. A Tukey post-hoc test showed that the antioxidant activity of the Soxhlet extract differed significantly from that of the other three methods.

These findings align with the determined levels of phenolics, flavonoids, and tannins, where higher concentrations correlate with stronger antioxidant activity. Phenolic compounds, flavonoids, and tannins are known to donate hydrogen atoms or electrons to neutralize free radicals. Therefore, the high content of secondary metabolites in the Soxhlet extract directly contributes to antioxidant activity (Muflihah *et al.*, 2021).

CONCLUSION

Soxhlet extraction produced the lowest IC₅₀ value (93.21 ppm) and the highest phenolic, flavonoid, and tannin contents, 92.48 mg GAE/g, 6.49 mg QE/g, and 97.85 mg TAE/g of extract, respectively, compared with maceration, percolation, and reflux extraction. These findings demonstrate differences in antioxidant activity and phytochemical content among the four extraction methods.

REFERENCES

- Attarde, D.L., Chaudhari, B.J., and Bhambar, R.S., (2011), Phytochemical Investigation and In Vitro Antioxidant Activity of Extracts from Leaves of *Limonia acidissima* Linn. (Rutaceae), *Journal of Pharmaceutical Research*, 4(3), pp. 766–768.
- Azizah, T.N., Harmastuti, N., & Wijayanti, T. (2024). Perbandingan Studi Aktivitas Antioksidan Ekstrak Metanol, Etanol 70%, dan Etanol 96% Daun Kawista (*Limonia acidissima* L.), *Jurnal Ilmu Farmasi dan Farmasi Klinik*, 21(1), pp. 56-64.
- Cao, S., Liang, J., Chen, M., Xu, C., Wang, X., Qiu, L., Zhao, X., Hu, W., (2025), Comparative Analysis of Extraction Technologies for Plant Extracts and Absolutes, *Frontiers in Chemistry*, 13, pp.1536590.
- Castillo-Correa, M., Montalbán-Hernández, C., Navarro-Hortal, M.D., Peña-Guzmán, D., Badillo-Carrasco, A., Varela-López, A., Hinojosa-Nogueira, D., and Romero Márquez, J.M., (2025), Exploring the Influence of Extraction Methods, Solvents, and Temperature on Total Phenolic Recovery and Antioxidant Capacity in Olive Leaf Extracts: A Systematic Review with Quantitative Synthesis. *Separations*, 12, pp. 236.
- Chandimali, N., Bak, S.G., Park, E.H., Lim, H.J., Won, Y.S., Kim, E.K., Park, S.I., and Lee, S.J., (2025), Free Radicals and Their Impact on Health and Antioxidant Defenses: a Review, *Cell Death Discovery*, 11(1), pp. 19.
- Fawwas, M., Pratama, M., Razak, R., Andika, Y., Astika, E., and Ferialia, (2024), Polyphenol Content and Antioxidant Evaluation of Kawista (*Limonia acidissima*) Leaf Extract, *Borneo Journal of Pharmacy*, 7(4), pp. 365-373.
- Febriana. N., Azhar, F., Abidin, Z, and Setyowati, D.N., 2024, Efektivitas Ekstrak Daun Kawista (*Limonia acidissima*) terhadap Sistem Imun Ikan Nila (*Oreochromis niloticus*) injection bacteria *Aeromonas hydrophila*
- Gulcin, İ., (2025), Antioxidants: a Comprehensive Review, *Archives of Toxicology*, 99(5), pp. 1893-1997.
- Hassanpour, S.H., and Doroudi, A., (2023), Review of the Antioxidant Potential of Flavonoids as a Subgroup of Polyphenols and Partial Substitute for Synthetic Antioxidants, *Avicenna Journal of Phytomedicine*, 13(4), pp. 354-376.

- Isnindar and Luliana, S., (2025), Effect of Extraction, Ratio, and Solvent Concentration on Total Flavonoid Content and Antioxidant Activity of Singkel (*Premna Serratifolia* Linn.) Using DPPH Method, *Jurnal Tekno Sains*, 15(2), pp. 103-114.
- Kalpoutzakakis, E., Chatzimitakos, T., Athanasiadis, V., Mitakou, S., Aligiannis, N., Bozinou, E., Gortzi, O., Skaltsounis, L.A., and Lalas, S.I., (2023), Determination of the Total Phenolics Content and Antioxidant Activity of Extracts from Parts of Plants from the Greek Island of Crete, *Plants (Basel)*, 12(5), pp. 1092.
- Martemucci, G., Costagliola, C., Mariano, M., D'andrea, L., Napolitano, P., and D'Alessandro, A.G., (2022), Free Radical Properties, Source and Targets, Antioxidant Consumption and Health, *Oxygen*, 2(2), pp. 48-78.
- Mufidah., Rohama., and Hidayah, N., (2025), Determination of Flavonoid Levels of Bidara Leaf Extract (*Ziziphus spina-christi* (L.) Desf) Based on Variation in Particle Size Using the UV-Vis Spectrophotometry Method, *As-Syifaa Jurnal Farmasi*, 17(2), pp. 145-152.
- Muflihah, Y.M., Gollavelli, G., AND Ling, Y.-C., (2021), Correlation Study of Antioxidant Activity with Phenolic and Flavonoid Compounds in 12 Indonesian Indigenous Herbs, *Antioxidants*, 10, pp. 1530.
- Mukhriani., (2014), Ekstraksi, Pemisahan Senyawa, dan Identifikasi Senyawa Aktif, *Jurnal Kesehatan*, 7(2), pp. 361-367.
- Nugroho, A., (2017), *Teknologi Bahan Alam*, Buku Ajar, Universitas Lambung Mangkurat, Banjarmasin.
- Nurhasnawati, H., Sukarmi, S., and Handayani, F., (2017), Perbandingan Metode Ekstraksi Maserasi dan Sokletasi terhadap Aktivitas Antioksidan Ekstrak Etanol Daun Jambu bol (*Syzygium malaccense* L.), 3(1), pp. 91-95.
- Pérez, M., Dominguez-López, I., and Lamuela-Raventós, R.M., (2023T), The Chemistry Behind the Folin-Ciocalteu Method for the Estimation of (Poly)phenol Content in Food: Total Phenolic Intake in a Mediterranean Dietary Pattern. *J Agric Food Chem*, 71(46):17543-17553.
- Rahimah, S.B., Djunaedi, D.D., Soeroto, A.Y., and Bisri, T., (2019). The Phytochemical Screening, Total Phenolic Contents and Antioxidant Activities in Vitro of White Oyster Mushroom (*Pleurotus Ostreatus*) Preparations, *Open Access Macedonia Journal of Medical Sciences*, 7(15), pp.2404-2412.
- Rauf, A.A., and Saranani, S. (2023). Penetapan Kadar Polifenol Total dan Tanin Total dari Ekstrak Etanol Buah Senggani (*Melastoma malabathricum* L.) serta Uji Aktivitas Antioksidan dengan Metode ABTS. *Jurnal Pharmacia Mandala Waluya*, 2(6), pp. 295–304.
- Reo, A.R., Berhimpion, S., and Montolalu, R., (2017), Secondary Metabolites of *Gorgonia*, *Paramuricea Clavata*, *Jurnal Ilmiah Platax*, 5(1), pp. 42–48.
- Ridwanuloh, D., and Mursal, I.L.P. (2018). Isolasi Metabolit Sekunder dari Daun Kawista (*Limonia acidissima* L.). *Pharma Xplore: Jurnal Ilmiah Farmasi*, 3(1), pp. 159-163.
- Safitri, I., Nuria, M.C., & Puspitasari, A.D., (2018), Perbandingan Kadar Flavonoid dan Fenolik Total Ekstrak Metanol Daun Beluntas (*Pluchea Indica* L.) pada Berbagai Macam Ekstraksi, *Jurnal Inovasi Teknik Kimia*, 3(1), pp. 31–36.
- Syamsudin, S., Alimuddin, A.H., & Sitorus, B., (2022), Isolasi dan Karakterisasi Senyawa Fenolik dari Daun Putat (*Planchonia Valida* Blume), *Indonesian Journal Of Pure And Applied Chemistry*, 5(2), 85-98.
- Verawati, V., Nofiandi, D., & Petmawati, P., (2017), Pengaruh Metode Ekstraksi terhadap Kadar Fenolat Total dan Aktivitas Antioksidan Daun Salam (*Syzygium polyanthum* (Wight) Walp.), *Jurnal Katalisator*, 2(2), pp. 53–60.
- Yudistirani, S.A., & Islam., M.B. (2019), Metode Ekstraksi untuk Perolehan Kandungan Flavonoid Tertinggi dari Ekstrak Daun Kelor'. *Jurnal Konversi*. 8(2), pp. 31–35.