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EFFECT OF EXTRACTION METHODS ON TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY POTENTIAL OF ETHANOL EXTRACT OF GALING-GALING LEAVES (*Cayratia trifolia* (L.) Domin)

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ABSTRACT

Background: Antioxidants play a key role in the prevention and treatment of various diseases. Flavonoids, a class of secondary metabolites, are known for their strong antioxidant properties. *Cayratia trifolia* (L.) Domin is an Indonesian endemic plant with medicinal potential, particularly as a natural antioxidant source. The extraction process is a critical step in isolating bioactive compounds and must be optimized to maximize flavonoid content and antioxidant activity. **Objective:** This study aims to evaluate the effect of different extraction methods—maceration, sonication, and Soxhlet extraction—on the total flavonoid content and antioxidant activity of ethanolic extracts of *C. trifolia* (L.) Domin leaves. **Methods**: This experimental study used three extraction techniques: maceration, sonication, and Soxhlet extraction. Total flavonoid content was determined using UV-Vis spectrophotometry and expressed as mg quercetin equivalent per gram of extract (mg QE/g extract). Antioxidant activity was measured using the DPPH free radical scavenging assay and expressed as IC_{50} (ppm). Statistical analysis was conducted using one-way ANOVA with a 95% confidence level. **Results:** The sonication method yielded the highest total flavonoid content (41.82 ± 0.011 mg QE/g extract) and the strongest antioxidant activity (IC_{50} = 66.523 ± 0.449 ppm), followed by Soxhlet extraction (35.78 ± 0.01 mg QE/g; IC_{50} = 84.217 ± 0.565 ppm), and maceration (18.48 ± 0.01 mg QE/g; IC_{50} = 94.579 ± 1.12 ppm). Statistical analysis showed that the extraction method significantly affected both total flavonoid content and antioxidant activity ($P < 0.05$). **Conclusion**: The extraction method significantly influences the yield of total flavonoids and antioxidant activity in *C. trifolia* ethanolic leaf extracts. Sonication was found to be the most effective method, suggesting its potential use in optimizing the recovery of antioxidant compounds from plant materials.

Keywords: Antioxidant Activity; *Cayratia trifolia* (L.) Domin; DPPH; Extraction methods; Total Flavonoid Contents.

INTRODUCTION

Antioxidants play a critical role in the prevention and treatment of several diseases, including cancer, cardiovascular disorders, cataracts, immune system decline, and neurodegenerative conditions[1]. Today, antioxidants are widely produced both synthetically and from herbal sources. However, synthetic antioxidants are often associated with adverse effects such as carcinogenicity, driving a growing interest in plant-based alternatives[2].

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Plants exhibit antioxidant activity primarily due to their secondary metabolites, including flavonoids, phenols, alkaloids, and tannins[3,4]. Flavonoids are among the most abundant classes of secondary metabolites, accounting for 5–10% of those found in plants. Their antioxidant potential arises from their hydroxyl groups, which can scavenge free radicals and donate hydrogen atoms to stabilize them[5].

Cayratia trifolia (L.) Domin, commonly known as galing-galing, has been traditionally used in herbal medicine for treating headaches, boils, wound healing, muscle pain and is mentioned in Balinese palm-leaf manuscripts *Usada Taru Pramana*[6,7]. Its leaves contain various bioactive compounds such as flavonoids, alkaloids, terpenoids, tannins, steroids, and saponins, with ethanol extracts reportedly containing total flavonoids up to 60.4 ± 0.45 mg/g[3]. Several studies have confirmed its antioxidant activity, with IC_{50} values ranging between 41.40 ± 0.64 mg/L and 74.83 mg/L depending on the extraction method and solvent[8].

Flavonoid content and antioxidant activity are influenced by both intrinsic (plant species, growing conditions) and extrinsic (processing) factors, with extraction being a critical step[9]. Extraction methods vary in solvent efficiency, thermal impact, and yield. Conventional methods include maceration, digestion, and Soxhlet, while modern approaches such as sonication, microwave-assisted, and supercritical fluid extraction offer improved efficiency[10]. Due to the thermal sensitivity of flavonoids—most degrade above 100°C—extraction method selection is vital. Maceration is simple and non-thermal, Soxhlet involves continuous extraction with minimal solvent use and indirect heating, while sonication accelerates extraction by facilitating cell wall disruption[11].

Previous studies on other plants have shown that extraction methods significantly affect both flavonoid content and antioxidant activity. For example, Fadlirarturahmah et al., found Soxhlet yielded the highest flavonoid levels in *Callicarpa longifolia*, while percolation produced the best antioxidant activity[12]. Das et al., reported that sonication provided superior results compared to maceration and Soxhlet[13]. To date, no study has compared the effect of different extraction methods on the total flavonoid content and antioxidant activity of *Cayratia trifolia* ethanol leaf extract. Therefore, this study aims to evaluate the impact of extraction method—maceration, Soxhlet, and sonication—on the total flavonoid content and antioxidant potential of *Cayratia trifolia* leaves.

METHODS

1. Types of Equipment and Materials

The instruments used were a blender (Philips) and mesh no. 60 sieve, analytical balance, glass jar, rotary evaporator (Buchii, Eyela®), porcelain crucible, filter paper, horn spoon, glass stirring rod, test tube, dropper pipette, graduated pipette, hot plate (Fischer Scientific®), porcelain crucible, oven (Binder®), Soxhlet apparatus set, sonicator (Branson), filter paper, vials and collection bottles (5 mL, 10 mL, 50 mL), stopwatch, UV-VIS spectrophotometer (SHIMADZU UV-2600).

C. trifolia (L.) Domin leaves were collected in Gianyar, Bali, Indonesia. The plant was identified at the Bali Botanic Garden, Indonesian Institute of Sciences. The solvents and reagents used in this study were as follows: ethanol (96%, technical grade), methanol (p.a., Merck®), distilled water (Bratachem®), deionized water, ash-free filter paper (Whatman™), hydrochloric acid (HCl, 10%, analytical grade, Merck®), hydrochloric acid (HCl, 2 N, analytical

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grade), sulfuric acid (H_2SO_4, 10%, analytical grade), acetic anhydride (analytical grade), aluminum chloride ($AlCl_3$, analytical grade, Merck®), sodium acetate (analytical grade), acetone (analytical grade), Mayer's reagent, Dragendorff's reagent, Wagner's reagent, silica gel GF254 TLC plates (Merck®), n-butanol (p.a., Merck®), acetic acid (p.a., Merck®), DPPH powder (analytical grade, Sigma®), and quercetin standard (analytical grade, Sigma®).

2. Extraction of *Cayratia trifolia* (L.) Domin Leaves

Fresh C. trifolia leaves were washed, chopped, blended, and dried at 50 °C for 24 hours, then ground into powder. Extraction was carried out using three methods: maceration (50 g powder with 500 mL 96% ethanol, repeated 3 times), Soxhlet extraction (25 g powder with 250 mL 96% ethanol at 76–80 °C), and sonication (25 g powder with 250 mL 96% ethanol for 60 minutes at 40 kHz, room temperature). All filtrates were concentrated using a rotary evaporator at 40 °C and further evaporated in a water bath to obtain crude extracts.

3. Phytochemical test

The extract is weighed at 5 mg, then dissolved in 10 mL ethanol. The tested compounds included alkaloids, flavonoids, triterpenoids, steroids, tannins, and saponins[14,15].

4. Total flavonoid content activity assay

1 mL of extract solution was mixed with 3 mL methanol (p.a.), 0.2 mL $AlCl_3$ 10%, 0.2 mL sodium acetate 1 M, and diluted to 10 mL with distilled water. After 30 minutes of incubation at room temperature, absorbance was measured at 428 nm using a UV-Vis spectrophotometer. Each sample was tested in six replicates, and total flavonoid content was calculated using a quercetin standard curve and expressed in mg QE/g extract using the Equation below:

$$Total\ Flavonoid\ Content = \frac{C.V.fp}{g}$$

Information C: Total flavonoid content; V: extract volume (mL); fp: dilution factor; g: sample weight (g).

5. DPPH radical scavenging activity assay

The DPPH radical scavenging activity test was carried out with a number of samples with 2 mL of DPPH 50 ppm and methanol added to 5 mL. The mixture was then vortexed and left for 30 minutes at room temperature, protected from light by wrapping in aluminium foil. The absorbance was measured at the maximum wavelenghth with the blank used being methanol. The percentage of inhibition (%IC) was determined by the following formula:

$$\%IC = \frac{Ax\text{-}As}{Ax}\ x\ 100\%$$

Information Ax: DPPH control absorbance and As:Sample absorbance. The value IC_{50} of each extract was calculated based on the linear regression equation y = bx + a resulting from a plot compare of sample concentration vs % radical scavenging.

6. Data Analysis

Total flavonoid content and IC_{50} values are reported as mean ± standard deviation. One-way ANOVA with a 95% confidence level (SPSS v27.0) was used to assess statistical significance. If significant ($p < 0.05$), analysis was followed by LSD post hoc test to identify differences between extraction methods.

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RESULTS

1. Phytochemical Screening.

Phytochemical screening was conducted to rapidly identify the classes of compounds present. The tests performed in this study included the detection of flavonoids, alkaloids, tannins, steroids and triterpenoids, as well as saponins. The results of the phytochemical screening of the extract are presented in Table 1.

2. Total flavonoid content in *C. trifolia* (L.) Domin Leaves

Each ethanolic extract of *C. trifolia* (L.) Domin leaves in different extraction methods has different total flavonoid content. The TFC for maceration, sonication, and Soxhlet extraction were 18.48 mgQE/g, 41.82 mgQE/g, and 35.78 mgQE/g, respectively (table 2). The TFC content in each extraction method was analyzed using one-way ANOVA to determine the significance of differences between the extraction methods. The results showed that there were statistically significant differences among the extraction methods. Subsequently, a post-hoc LSD test was conducted to identify specific group differences. Based on the post-hoc LSD results, each extraction method was found to produce statistically significant differences. Detailed results are presented in Table 3.

Table 1. Phytochemical screening on each extract of *C. trifolia* (L.) Domin

No	Phytochemical groups	Reagents	Positive results	Extraction methods		
				Maceration	Sonication	Soxhlet extraction
1	Flavonoid	Boric acid and oxalic acid	Intense yellow fluorescence under UV 366 nm or pink fluorescence with intense yellow	(+)	(+)	(+)
2	Tannin	FeCl3	A green-coloured solution or precipitate	(-)	(-)	(-)
3	Steroid/terpenoid	Liebermann-Burchard	Steroids: A bluish-green ring appeared at the interface of the solution	(+)	(+)	(+)
			Triterpenoids: A violet or brownish ring appeared at the interface of the solution.	(-)	(-)	(-)
4	Alkaloid	Mayer	Yellow precipitate	(+)	(+)	(+)
		Wagner	Red-brown precipitate	(+)	(+)	(+)
		Dragendroff	Red precipitate	(+)	(+)	(+)
5	Saponin	Hot water and HCl 2N	A stable foam with a height of 1–10 cm was formed and persisted for 10 minutes, even after the addition of 2N HCl.	(-)	(-)	(-)

Table information: (+): contain tested phytochemicals; (-): absence tested phytochemicals

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Table 2. Total flavonoid content on each extract C.trifolia (L.) Domin

Extraction Methods	TFC (mgQE/g) (mean+SD)	p-value
Maceration	18.48 ± 0.01	0.000*
Sonication	41.82 ± 0.01	
Soxhlet extraction	35.78 ± 0.01	

Table information: *indicating significant differences using One-Way ANOVA Test (p-value<0.05)

Table 3. Post-hoc LSD test of total flavonoid content

Extraction Methods	p-value		
	Maceration	Sonication	Soxhlet extraction
Maceration		0.000*	0.000*
Sonication			0.000*
Soxhlet extraction			

Table information: *****indicating significant differences using post-hoc LSD Test **(p-value<0.05)**

3. Antioxidant activity in *C. trifolia* (L.) Domin Leaves

Each ethanolic extract of *C. trifolia* (L.) Domin leaves obtained through different extraction methods exhibited different IC_{50} values, but all demonstrated the same antioxidant activity category, namely strong (Table 4). The extract obtained via sonication exhibited a stronger IC_{50} value compared to those obtained through Soxhlet and maceration methods (66.523 ± 0.449, 84.217 ± 0.565, and 94.579 ± 1.12, respectively). Statistical analysis showed that the extraction method had a statistically significant effect on the IC_{50} values ($p < 0.05$). Subsequently, a post hoc LSD test was conducted to identify specific group differences. Based on the post hoc LSD results, each extraction method was found to produce statistically significant differences. Detailed results are presented in Table 5.

Table 4. Antioxidant activity on each extract of *C.trifolia* (L.) Domin

Extraction Methods	IC_{50} (ppm) (Mean SD	p-value	Antioxidant Activity
Maceration	94.579 ± 1.12	0.000*	Strong
Sonication	66.523 ± 0.449		Strong
Soxhlet extraction	84.217 ± 0.565		Strong

Table information: *indicating significant differences using One-Way ANOVA Test (p-value<0.05)

Table 5. Post-hoc LSD test of antioxidant activity

Extraction Methods	p-value		
	Maceration	Sonication	Soxhlet extraction
Maceration		0.000*	0.000*
Sonication			0.000*
Soxhlet extraction			

Table information: *indicating significant differences using post-hoc LSD Test (p-value<0.05)

DISCUSSION

In this study, we reported the characteristics of extracts obtained through different extraction methods, as previously described. Flavonoid compounds were extracted in higher amounts using the sonication method compared to Soxhlet and maceration methods. This finding is supported by the review of Chavez-Gonzalez et al., which noted that sonication enhances the extraction of flavonoid compounds such as rutin, narcissin, nicotiflorin, epicatechin gallate, catechin, procyanidin B2, quercetin, and kaempferol[11].

The efficiency of the sonication method can be attributed to its mechanism, which involves ultrasonic waves at frequencies above 20 kHz (typically 40 kHz). These waves disrupt plant cell walls, facilitating the release of intracellular compounds. This process, driven by cavitation, generates mechanical and thermal energy, leading to reduced particle size and improved solvent–matrix interaction. Previous studies have shown that ultrasonic waves can enhance the

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release of flavonoids bound tightly to solid matrices and increase the rate of molecular diffusion [16,17].

Soxhlet extraction employs a temperature of 76 °C, aligning with the boiling point of ethanol. Heat energy reduces surface tension and viscosity, improving solvent penetration into plant matrices[18]. However, heat-sensitive flavonoid compounds may degrade under such conditions, leading to lower flavonoid content. For example, mesquitol and luteolin degrade by approximately 50% below 76 °C, while other compounds such as rutin, quercetin, naringin, and eriodyctiol are more heat-stable[19]. The maceration method yielded the lowest total flavonoid content, likely due to the absence of external forces that enhance solvent penetration. This method relies solely on the polarity of the solvent and manual stirring, performed 15 times daily, which may limit the diffusion of active compounds from the plant matrix[20].

Antioxidant activity, measured as IC_{50}, showed that the sonicated ethanolic extract had the strongest potential (66.523 ± 0.449 ppm), followed by Soxhlet (84.217 ± 0.565 ppm) and macerated extracts (94.579 ± 1.12 ppm). Quercetin, used as a standard, exhibited very strong antioxidant activity (4.882 ± 0.06 ppm). All extracts fall under the “strong” category (IC_{50} between 50–100 ppm), while quercetin is categorized as “very strong” ($IC_{50} < 50$ ppm). These results are consistent with findings from Junior et al. (2024), who reported strong antioxidant activity in pure ethanol extracts of *C. trifolia* leaves (IC_{50} with DPPH test is 5.03± 0.2 ppm[21]. The extraction results using infusion and decoction methods on *C. trifolia* (L.) Domin also yielded IC_{50} values categorized as strong (87.55 ± 1.01 and 65.38 ± 2.26 µg/mL, respectively)[7]. The difference in antioxidant activity among extraction methods involving heating, such as infusion, decoction, and Soxhlet extraction, is influenced by the duration of heating, where longer heating can enhance the solvent's kinetic properties; however, excessive heating may lead to the degradation of thermolabile compounds[22].

Flavonoids have a mechanism of capturing free radicals (hydroxyl radicals, superoxide, and peroxyl) and inhibiting various oxidation reactions because they can produce phenolic radicals, which are stabilized by the resonance effect of aromatic rings[23]. This study shows the correlation between TFC and antioxidant activity, where a higher flavonoid content corresponds to stronger antioxidant activity.

Overall, the sonication method produced extracts with the highest antioxidant activity. This is likely due to the enhanced mass transfer and surface disruption enabled by ultrasonic waves, which facilitate more efficient extraction of antioxidant compounds. Yang et al. similarly observed increased antioxidant activity in blueberries extracted by sonication, attributed to elevated levels of flavonoids and polyphenols[24]. In contrast, the Soxhlet method may cause thermal degradation of heat-sensitive antioxidant compounds, while the maceration method, lacking thermal or ultrasonic stimulation, results in the lowest antioxidant yield. Supporting this, Elya et al. demonstrated that Soxhlet extraction of *Rubus fraxinifolius* produced higher DPPH inhibition than maceration[25]. The results of this study highlight the importance of selecting an appropriate extraction method based on the characteristics of the plant material and the target compounds, particularly in the context of industrial pharmacy.

CONCLUSION

The present study demonstrated that the extraction method significantly influences the total flavonoid content and antioxidant

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activity of *Cayratia trifolia* (L.) Domin leaf ethanolic extracts. Among the three methods tested, sonication, Soxhlet extraction, and maceration, sonication yielded the highest total flavonoid content and the strongest antioxidant activity, as indicated by the lowest IC_{50} value. This can be attributed to the cavitation effect of ultrasonic waves, which enhances cell wall disruption and mass transfer efficiency. In contrast, the Soxhlet method, although more efficient than maceration, may cause partial degradation of heat-sensitive flavonoids due to prolonged exposure to elevated temperatures. The maceration method yielded the lowest flavonoid content and antioxidant activity, likely due to the absence of external energy input. These findings highlight the importance of selecting an appropriate extraction method to optimize the yield of bioactive compounds, particularly flavonoids, for their potential antioxidant benefits.

CONFLICT OF INTEREST

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