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Research Article

Polyphenol Content and Antioxidant Evaluation of Kawista (*Limonia acidissima*) Leaf Extract

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Abstract

Kawista or *Limonia acidissima*, a plant traditionally used by the Bima community, offers potential health benefits. This study aimed to evaluate the antioxidant potential of ethanol extract from *L. acidissima* leaves by determining total flavonoid content (TFC), total phenolic content (TPC), and antioxidant activity using DPPH, FRAP, and CUPRAC assays. The ethanol extract exhibited significant antioxidant activity, with IC_{50} values of 10.445 and 135.42 µg/mL for DPPH and CUPRAC assays, respectively. TPC and TFC were determined to be 14.63 mgGAE/g extract and 113.9 mgQE/g extract, respectively. These findings suggest that *L. acidissima* leaf extract possesses potent antioxidant properties, which may be attributed to its flavonoid and phenolic content. Further research is warranted to explore this plant extract's underlying mechanisms of action and potential therapeutic applications.

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INTRODUCTION

Antioxidants are vital compounds that act as a defense mechanism against oxidative stress by neutralizing harmful reactive oxygen species (ROS) in the body[1]. These compounds can be classified as endogenous, produced within the body, or exogenous, obtained from external sources. Exogenous antioxidants are abundant in natural sources such as fruits, vegetables, and whole grains[2]. Recognizing the importance of natural antioxidants, this study focuses on the kawista (*Limonia acidissima*) fruit, a plant traditionally used in Bima Regency, Indonesia, to treat various ailments.

Previous studies have demonstrated that *L. acidissima* fruit possesses antioxidant properties, and it is traditionally utilized as a fever reducer, tonic, and remedy for stomach ailments. Furthermore, the fruit skin of *L. acidissima* has also been shown to exhibit antioxidant activity, attributed to the presence of various phytochemicals such as phenolic compounds, flavonoids, steroids, saponins, tannins, and alkaloids[3]. These secondary metabolites possess antioxidant properties due to their ability to donate electrons to neutralize free radicals, effectively stabilizing these reactive species[4]. Beyond its antioxidant potential, *L. acidissima* fruit has shown promise as an anti-diabetic agent, while the leaves have been reported to exhibit hepatoprotective properties[3].

Building upon these empirical observations, this research will investigate the phytochemical profile of *L. acidissima* leaves. Specifically, this study aims to quantify the levels of total flavonoids and phenolics in ethanolic extracts of *L. acidissima* leaves and subsequently evaluate their antioxidant potential. This research will contribute to a better understanding of the phytochemical composition and potential health benefits of *L. acidissima* leaves. Previous studies have employed various methods to evaluate antioxidant potential, including radical scavenging assays[5,6]. This study investigated the antioxidant activity of ethanolic extracts of *L. acidissima* leaves using three complementary assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH)

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radical scavenging, ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC). All assays were conducted using UV-visible spectrophotometry for quantitative analysis.

MATERIALS AND METHODS

Materials

This study employed analytical instruments including micropipettes (OneMed®), an oven (Memmert®), a pH meter (Jenco®), a centrifuge (OneMed®), a UV-visible spectrophotometer (Aple®), an analytical weighing scale (KERN®), and a vortex mixer (IKA® Vortex Genius 3). All glassware used was of analytical grade (Pyrex®). Analytical-grade chemicals and reagents were procured from commercial sources. Fresh leaves of *L. acidissima* (**Figure 1**), obtained from Bima Regency, Indonesia, were used in this study. The botanical identification of the plant material (specimen No. KW.001.13032024) was confirmed by the Division of Botany, Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Muslim Indonesia, Makassar, Indonesia. The reference standards, including quercetin, ascorbic acid, and gallic acid, were obtained from Merck Co. (Darmstadt, Germany). Reagents for antioxidant evaluation, such as DPPH, FRAP, and CUPRAC reagents, were purchased from Sigma-Aldrich (Singapore). Deionized water was produced using a Millipore-Q50 Ultrapure water system (Sartorius). Stock solutions of the reference standards (1000 µg/mL) were prepared by dissolving 10 mg of each standard in 10 mL of ethanol.

Figure 1. *Limonia acidissima* leaves from Bima Regency.

Methods

Extraction

Fresh leaves of *L. acidissima* were meticulously washed with deionized water and subsequently air-dried under ambient conditions. After thorough drying, the leaves were meticulously sorted and then meticulously cut into small pieces using a clean, sterile instrument. The powdered leaf material was obtained by grinding the dried leaf pieces to a fine powder using

a suitable grinder. A total of 100 g of this powdered material was subjected to maceration using 1000 mL of 96% ethanol at room temperature for a duration of three days. The resulting ethanolic extract was meticulously filtered to remove any particulate matter and subsequently concentrated under reduced pressure using a rotary evaporator. The concentrated extract was further dried in a vacuum desiccator to obtain a dry extract. The yield of the extraction process was calculated using **Equation 1**.

$$\%yield = \frac{Total\ weight\ of\ extract\ (g)}{Total\ weight\ of\ sample\ (g)} \text{x} 100\% \quad [1]$$

Qualitative analysis of flavonoids and phenolics

Initially, 5 mg of ethanolic extracts of *L. acidissima* leaves were mixed with 96% ethanol to prepare a stock solution. This mixture was then heated and subsequently filtered. To detect the presence of flavonoids, 1 mL of concentrated HCl, 1 mL of amyl alcohol, and magnesium metal were added to the extract solution. The formation of a yellow color indicated the presence of flavonoids. Furthermore, the presence of phenolic compounds was confirmed by adding 1% $FeCl_3$ to the extract solution. A green-to-black coloration was observed as a positive indicator for phenolics[7].

Determination of total flavonoid content (TFC)

The TFC of the ethanolic extracts of *L. acidissima* leaves was determined using the aluminum chloride colorimetric method, adapted from previously published protocols[8,9]. A standard curve was generated using varying concentrations of quercetin (6, 8, 10, 12, and 14 µg/mL). To each 1 mL quercetin concentration, 1 mL of 2% $AlCl_3$ and 1 mL of 120 mM potassium acetate were added. The mixtures were incubated at room temperature for 30 minutes, followed by absorbance measurement at 449 nm using a UV-Vis spectrophotometer. A linear regression analysis ($y = bx + a$) was performed to determine the best fit for the quercetin standard curve.

Determination of total phenolic content (TPC)

The TPC of the ethanolic extracts of *L. acidissima* leaves was determined using the Folin-Ciocalteu method with slight modifications[10]. A standard curve was prepared using various concentrations of gallic acid (6, 8, 10, 12, and 14 µg/mL). To each 1 mL gallic acid standard, 1 mL Folin-Ciocalteu reagent was added, followed by 1 mL 7% sodium carbonate. After 5 minutes of incubation at room temperature, the absorbance was measured at 752 nm using a UV-Vis spectrophotometer. The absorbance values were plotted against gallic acid concentrations, and a linear regression equation was generated to obtain the standard curve.

Evaluation of antioxidant activity by DPPH assay

The antioxidant activity of the ethanolic extracts of *L. acidissima* leaves was determined using the DPPH radical scavenging assay with slight modifications[6,11]. Quercetin (1000 µg/mL) was used as a positive control and diluted to prepare a concentration series (1, 2, 3, 4, 5, and 6 µg/mL). Briefly, 1 mL of each quercetin concentration or ethanolic extracts of *L. acidissima* leaves (10, 20, 30, 40, and 50 µg/mL) was mixed with 4 mL of DPPH solution (35 µg/mL) in a glass test tube. The mixture was transferred to a disposable polystyrene cuvette and incubated at 37°C for 30 minutes. The absorbance of the reaction mixture was measured at 517 nm using a UV-visible spectrophotometer. A blank sample containing methanol (2 mL) and DPPH solution (1 mL) was prepared similarly. All samples were prepared in triplicate. To minimize photodegradation of the DPPH radical, all samples were protected from light until analysis[6,11].

Evaluation of antioxidant activity by FRAP assay

Antioxidant activity was determined using the FRAP assay, modified from previously published methods[9,12]. Briefly, a standard curve was generated using quercetin at concentrations of 10, 15, 20, 25, and 30 µg/mL. Each quercetin standard (1 mL) was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6), 1 mL of potassium ferricyanide, and incubated at 50°C for 20 minutes. Subsequently, 1 mL of trichloroacetic acid, 1 mL of deionized water, and 0.5 mL of ferric chloride were added to the mixture. After centrifugation at 3000 rpm for 10 minutes, the absorbance of the supernatant was measured at 744 nm using a UV-visible spectrophotometer. The same procedure was performed in triplicate for the ethanolic extracts of *L. acidissima* leaves at a concentration of 1000 µg/mL. The absorbance values from the quercetin standard curve were used to construct a linear regression equation.

Evaluation of antioxidant capacity by CUPRAC assay

The antioxidant capacity of the ethanolic extracts of *L. acidissima* leaves was determined using the CUPRAC assay with slight modifications[13]. Ascorbic acid served as the positive control. A stock solution of 1000 µg/mL ascorbic acid was prepared by dissolving 2.5 mg of ascorbic acid in 1% oxalic acid. Serial dilutions were then made to obtain concentrations of 100, 150, 200, 300, and 350 µg/mL. To each concentration of ascorbic acid and the ethanolic extracts of *L. acidissima* leaves (50, 100, 150, 200, 300, and 400 µg/mL), 1 mL of neocuproine reagent, 1 mL of ammonium acetate buffer (pH 7), and 1 mL of ethanol were added. The mixtures were incubated for 30 minutes at room temperature. Subsequently, the absorbance of each sample was measured at 450 nm using a UV-Visible spectrophotometer. In the CUPRAC assay, antioxidant compounds reduce Cu^{2+} to Cu^{+}, which then forms a chromophore with neocuproine, resulting in the formation of a yellow-colored complex.

Data analysis

The TFC of the ethanolic extracts of *L. acidissima* leaves was determined by plotting its absorbance on the quercetin standard curve and expressing the result as mg of quercetin equivalents (mgQE) per g of extract (mgQE/g) using **Equation 2**. This procedure was repeated three times for each ethanolic extracts of *L. acidissima* leaves sample to ensure reproducibility. The TPC of the ethanolic extracts of *L. acidissima* leaves was determined by measuring the absorbance of the ethanolic extracts of *L. acidissima* leaves samples in triplicate and interpolating the values onto the gallic acid standard curve. The results were expressed as mg of gallic acid equivalents (mgGAE) per g of extract (mgGAE/g) using **Equation 3**[9,10].

$$Total\ flavonoid\ content\ (mgQE/g) = \frac{Sample\ volume\ (L) \times Initial\ concentration\ (mg/L)}{Extract\ weight\ (g)} \quad [2]$$

$$Total\ phenolic\ content\ (mgGAE/g) = \frac{Sample\ volume\ (L) \times Initial\ concentration\ (mg/L)}{Extract\ weight\ (g)} \quad [3]$$

The percentage of DPPH radical scavenging activity was calculated using the following **Equation 4**, in which ACO is the absorbance of the control (DPPH solution alone) at t=0 and AAT is the absorbance of the sample at t=30 minutes. The antioxidant activity of ethanolic extracts of *L. acidissima* leaves was expressed as mgQE/g using the following **Equation 5**. The antioxidant activity of the ethanolic extracts of *L. acidissima* leaves was expressed as the EC_{50} value, which represents the concentration of the extract required to inhibit the reduction of Cu(II) by 50%. The EC_{50} values were determined from the calibration curve generated using the ascorbic acid standards[14] and calculated using **Equation 6**.

$$\%inhibition = \frac{(A_{CO} - A_{AT})}{A_{CO}} \text{x} 100\% \quad [4]$$

$$Antioxidant\ capacity\ (mgQE/g) = \frac{Sample\ volume\ (L) \times Initial\ concentration\ (mg/L)}{Extract\ weight\ (g)} \quad [5]$$

$$\%capacity = (1 - T)\ \text{x}\ 100\% \quad [6]$$

RESULTS AND DISCUSSION

Extraction of *L. acidissima* leaves was performed using the maceration method with 96% ethanol. A yield of 11.83% (w/w) of ethanolic extract of *L. acidissima* leaves was obtained from 200 g of dried leaf powder. Qualitative phytochemical screening revealed the presence of flavonoids, evidenced by the formation of a yellow color upon reaction with $AlCl_3$. Furthermore, the presence of phenolic compounds was confirmed by the green color reaction with $FeCl_3$. Quantitative analysis of TFC and TPC was conducted by measuring the absorbance of the ethanolic extract of *L. acidissima* leaves and comparing the values to standard curves generated from quercetin and gallic acid, respectively. The absorbance data and the corresponding TFC and TPC levels are summarized in **Table I**.

The DPPH radical scavenging assay is a well-established and widely utilized method for evaluating the antioxidant capacity of various compounds, including flavonoids, ascorbic acid, peptides, and phenolic compounds[5]. This method leverages the stable free radical nature of DPPH, offering several advantages such as simplicity, cost-effectiveness, and rapid analysis compared to other antioxidant assays. In its stable form, DPPH exhibits a characteristic deep purple color. However, upon

interaction with an antioxidant compound, DPPH undergoes reduction, resulting in a color change from deep purple to yellow[15]. This color change signifies the antioxidant's ability to donate either a hydrogen atom or an electron to the DPPH radical[16].

Spectrophotometry is a well-established technique for assessing color changes and evaluating antioxidant activity[17]. In this study, the DPPH radical scavenging activity was measured at 515 nm, its maximum absorbance wavelength. The IC_{50} value, representing the concentration of the extract required to inhibit 50% of DPPH radicals, was determined to quantify antioxidant potency. A lower IC_{50} value generally indicates higher antioxidant activity[18]. Based on established criteria, antioxidant activity can be categorized as very strong (IC_{50} <50 µg/mL), strong (50-100 µg/mL), moderate (100-250 µg/mL), and weak (250-500 µg/mL)[19]. The ethanolic extract of *L. acidissima* leaves exhibited potent antioxidant activity with an IC_{50} value of 10.445 µg/mL, classifying it as a very strong antioxidant. However, quercetin, a potent antioxidant flavonoid, demonstrated slightly higher antioxidant activity with an IC_{50} of 6.207 µg/mL, as shown in **Table II**. This observation is expected, considering quercetin is a pure compound with well-documented antioxidant properties. A previous study reported that *L. acidissima* fruit possesses antioxidant activity, exhibiting an IC_{50} value of 41.35 g/mL in a DPPH radical scavenging assay[20]. These findings suggest that the antioxidant potential of ethanolic extract of *L. acidissima* leaves surpasses that of *L. acidissima* fruit, indicating a promising avenue for further exploration of its potential as a natural antioxidant.

Table I. TFC and TPC level of ethanolic extract of *L. acidissima* leaves.

Analysis	Absorbance (449 nm)	Linearity regression	Initial concentration (mg/L)	TFC (mgQE/g)/ TPC (mgGAE/g)	Average (mgQE/g)/ (mgGAE/g)
TFC	0.326	$y = 0.0132x + 0.148$ (r = 0.990)	35.909	118.4	113.9
	0.333		36.439	115.6	
	0.306		34.393	107.0	
TPC	0.225	$y = 0.067x - 0.2621$ (r = 0.998)	7.270	14.25	14.63
	0.264		7.852	14.72	
	0.266		7.882	15.01	

Table II. Antioxidant activity of ethanolic extract of *L. acidissima* leaves by DPPH assay.

Analysis	Concentrations (µg/mL)	Absorbance (516 nm)	Inhibition (%)	IC_{50} (µg/mL)
Quercetin	1	0.668	26.187	6.207
	3	0.578	36.132	
	4	0.553	38.895	
	5	0.52	42.541	
	6	0.497	45.082	
Ethanolic extract of *L. acidissima* leaves	10	0.807	1.328	10.445
	20	0.79	3.207	
	30	0.671	16.356	
	40	0.589	25.417	
	50	0.452	40.555	

The antioxidant activity of ethanolic extract of *L. acidissima* leaves was evaluated using the FRAP assay. This assay measures the ability of the extract to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The results, presented in **Table III**, demonstrate that ethanolic extract of *L. acidissima* leaves possesses significant antioxidant capacity, with an average value of 473.41 mgQE/g. This value is comparable to the antioxidant capacity of quercetin itself, as evidenced by the linear regression analysis of quercetin ($y = 0.0088x + 0.1204$, r = 0.997). Free radicals, characterized by unpaired electrons, are highly reactive species that can readily interact with cellular macromolecules such as lipids, proteins, and DNA, leading to oxidative damage. These reactive species are generated during normal cellular metabolism and can also be produced in response to external factors like air pollution and ultraviolet radiation[21,22]. The observed antioxidant activity of ethanolic extract of *L. acidissima* leaves can be attributed to the presence of polyphenols, which have been well-documented to possess strong antioxidant properties[23,24]. This finding suggests that ethanolic extract of *L. acidissima* leaves may have potential as a natural source of antioxidants for various applications.

The CUPRAC assay was employed to evaluate the antioxidant capacity of ethanolic extract of *L. acidissima* leaves by assessing its ability to reduce Cu^{2+} to Cu^{+} through free radical scavenging[25]. This method offers several advantages, including high selectivity, cost-effectiveness, and stability compared to other colorimetric assays. Notably, the CUPRAC assay is not susceptible to interference from environmental factors such as air, moisture, or sunlight[26]. Ascorbic acid served as a positive control in this study. The EC_{50} value for ethanolic extract of *L. acidissima* leaves was determined to be 135.42

µg/mL, which was significantly higher than that of ascorbic acid, indicating lower antioxidant activity. The detailed antioxidant activity of ethanolic extract of *L. acidissima* leaves and ascorbic acid is presented in **Table IV**.

Table III. Antioxidant activity of ethanolic extract of *L. acidissima* leaves by FRAP assay.

Analysis	Concentrations (µg/mL)	Absorbance (744 nm)	Antioxidant capacity (mgQE/g)	Average antioxidant capacity (mgQE/g)
Quercetin	10	0.214	-	-
	20	0.25	-	
	30	0.289	-	
	40	0.346	-	
	50	0.387	-	
Ethanolic extract of *L. acidissima*	1000	0.561	500.68	473.41
leaves	1000	0.564	504.09	
	1000	0.486	415.45	

Table IV. Antioxidant activity of ethanolic extract of *L. acidissima* leaves by CUPRAC assay.

Analysis	Concentrations (µg/mL)	Absorbance (516 nm)	Inhibition (%)	IC_{50} (µg/mL)
Ascorbic acid	350	0.594	73.023	20.43
	300	0.485	65.236	
	200	0.317	48.95	
	150	0.26	33.319	
	100	0.205	23.264	
Ethanolic extract of *L. acidissima* leaves	50	0.318	23.264	135.42
	100	0.298	46.912	
	150	0.42	59.913	
	200	0.541	69.661	
	300	0.663	77.091	
	400	0.799	83.251	

Limited data exists on the antioxidant activity of ethanolic extract of *L. acidissima* leaves. Therefore, this study compared the antioxidant activity of *L. acidissima* leaf extracts with that of *L. acidissima* fruit. Previous research has demonstrated antioxidant potential in *L. acidissima* fruit, with an IC_{50} value of 30.28 µg/mL determined using the ABTS method[20]. These findings suggest that *L. acidissima* fruit exhibits higher antioxidant activity compared to the leaf extracts evaluated in the present study.

CONCLUSION

The current study demonstrated that the ethanolic extract of *L. acidissima* leaves exhibits potent antioxidant activity, as evidenced by its strong scavenging ability against DPPH radicals. This antioxidant activity was comparable to that of quercetin, a well-known flavonoid with potent antioxidant properties. Further supporting these findings, the CUPRAC and FRAP assays also revealed significant antioxidant capacity of ethanolic extract of *L. acidissima* leaves. These results collectively suggest that ethanolic extract of *L. acidissima* leaves holds considerable promise as a potential source of natural antioxidants. However, further *in vivo* studies are crucial to validate these findings and assess the potential therapeutic applications of ethanolic extract of *L. acidissima* leaves.

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AUTHORS' CONTRIBUTION

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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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