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Original Article

EVALUATION OF TOTAL PHENOLIC CONTENT, ANTIOXIDANT, AND ANTI-INFLAMMATORY POTENTIAL OF *MURRAYA KOENIGII*

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ABSTRACT

Objective: This study aims to determine the total phenolic and flavonoid content and evaluate the *in vitro* antioxidant and anti-inflammatory activity of *Murraya koenigii* leaf extract.

Methods: Ethanolic extract of *M. koenigii* leaves was prepared using the remaceration extraction process. Antioxidant activity was assessed using DPPH free radical scavenging, ABTS, and FRAP tests. Anti-inflammatory activity was evaluated *in vitro* using the protein denaturation method.

Results: *M. koenigii* leaf extract contained a total phenolic content of 211.7329 GAE and a total flavonoid content of 157.2957 QA. The extract exhibited strong antioxidant activity based on DPPH, ABTS, and FRAP tests, with IC_{50} values of 28.82 mg/l, 19.02 mg/l, and 30.68 mg/l, respectively. Additionally, the extract significantly inhibited protein denaturation, demonstrating anti-denaturation activity with an IC_{50} of 19.65 µg/ml, compared to diclofenac sodium, which had an IC_{50} of 11.34 µg/ml.

Conclusion: These findings suggest that *M. koenigii* leaf extract possesses strong antioxidant and anti-inflammatory properties, making it a potential adjunctive treatment for inflammation-related pain. Future studies elucidating the underlying mechanisms of these effects could have significant implications for clinical science.

Keywords: Anti-inflammatory, Antioxidant, Curry leaf, Ethanolic extract, Phenolic acids

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INTRODUCTION

Inflammation is a biological response triggered by harmful stimuli. During the inflammatory response, the immune system activates immune cells and releases pro-inflammatory mediators, leading to changes in the homeostatic environment and contributing to disease progression. Inflammation activates various types of cells that secrete inflammatory markers, such as prostaglandins (PGE2), cytokines (TNF-α, IL-1β, IL-6), and nitric oxide (NO) [1]. Modern human diseases are almost invariably associated with inflammation [2].

The inflammatory process involves increased vascular permeability, protein denaturation, and membrane alterations. Protein denaturation is a process in which proteins lose their tertiary and secondary structures due to exposure to external factors such as strong acids and bases, high concentrations of inorganic salts, organic solvents, and heat [3]. In general, denatured proteins will lose their biological function.

Mild inflammation can help stimulate the immune system to eliminate pathogens, metabolites, necrotic tissue, inflammatory factors, and other potentially harmful substances while supporting metabolic processes. However, if left uncontrolled, inflammation can disrupt normal bodily functions [4]. When inflammation occurs, it is often accompanied by pain and oxidative stress. Therefore, anti-inflammatory treatment may also relieve pain and reduce oxidative stress [5]. If inflammation worsens or is not controlled, tissue damage can occur, ultimately affecting quality of life and work efficiency.

Antioxidants can mitigate these negative effects by scavenging free radicals. This activity includes scavenging *2,2-diphenyl-1-picrylhydrazyl* (DPPH) free radicals, hydrogen peroxide (H_2O_2), *2,2′-azino-bis*(*3-ethylbenzothiazoline-6-sulfonic acid*) (ABTS), and hydroxyl (OH) radicals, as well as the activity of antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase [6]. In this context, natural antioxidants are the preferred choice due to their abundance, low cost, and fewer side effects. Flavonoids and phenolics are secondary metabolites found in plants that contribute to their antioxidant potential [7]. Phenolic substances can also block pro-inflammatory mediators other than COX by preventing their activity or gene expression. Additionally, certain phenolic compounds have the ability to alter the expression of transcriptional factors involved in inflammatory and antioxidant pathways, such as nuclear factor-kB (NF-kB) or Nrf-2 [8].

To reduce inflammation, nonsteroidal anti-inflammatory drugs (NSAIDs), such as sodium diclofenac, indomethacin and ibuprofen, are commonly used, while both acute and chronic inflammation can be treated with non-opioid analgesics like aspirin [9]. However, long-term use of these drugs may lead to adverse side effects, including respiratory depression, gastrointestinal disturbances, kidney damage, and dependency [10]. Consequently, researchers are currently seeking new anti-inflammatory agents that do not pose these risks [11]. Ongoing studies aim to discover novel therapeutic compounds from medicinal plants that can be used to suppress inflammation, one of which is *Murraya koenigii*.

Murraya koenigii Linn. (Rutaceae), commonly known as curry leaf, has been identified to contain phenolic acids, including tannic, gallic, vanillic, cinnamic, ferulic, and caffeic acids [12]. In traditional medicine, *M. koenigii* is used as a stomachic, antipyretic, anti-inflammatory, antidiarrheal, tonic, and remedy for dysentery and nausea, and as a blood purifier [13–16]. In this study, the antioxidant potential *of M. koenigii* was evaluated using the DPPH, ABTS, and FRAP methods. The total phenolic and flavonoid content was also analyzed. Additionally, anti-inflammatory activity tests were also carried out using the *in vitro* protein denaturation inhibition method, measured with a UV-Vis spectrophotometer. Diclofenac has also been shown to possess superior intrinsic anti-inflammatory activity compared to other NSAIDs. Therefore, diclofenac was used

as the standard in this study. This study aims to examine total polyphenols and flavonoids contents along with antioxidant and anti-inflammatory activity of *M. koenigii* extract.

MATERIALS AND METHODS

Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS ([2,2′ azino-bis (3-ethylbenzothiazoline 6-sulfonic acid)]), ammonium molybdate, $FeCL_3$, gallic acid, quercetin, Folin Ciocalteu, aluminum chloride, ascorbic acid, bovine serum albumine, and diclofenac sodium were purchased from Sigma Aldrich (USA). All additional chemicals and solvents utilized were of analytical grade (Merck).

Plant material and extract preparation

M. koenigii leaves were collected in May 2023 from Klipang, Semarang, Central Java, Indonesia. The plant material was identified by a botanist at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Indonesia. The plants were authenticated using voucher specimens for *M. koenigii* (45.25.9/UNI/FFA.2/BF/PT/2023) deposited at the Faculty of Pharmacy, Gadjah Mada University, Indonesia.

The plant material was dried under sunlight. The dried sample was then ground using a laboratory grinder and sieved through a 40-mesh sieve. A total of 500 g of *M. koenigii* dried leaf powder was mixed with 5 l of 90% ethanol. The extraction process was carried out using the maceration method, repeated three times for 24 h each at room temperature. The obtained filtrate was collected and concentrated at 70 °C using a rotary evaporator to obtain *Murraya koenigii* extract (MKE). The yield of the extract was 22.78%w/w.

The amount of phenolic compounds and flavonoids

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method [17], while the total flavonoid content (TFC) in the plant extract was measured using the aluminum chloride colorimetric method [18]. The TPC value was expressed in mg of gallic acid equivalent (GAE) per g of dry extract, while the TFC of the extracts was determined as mg of quercetin equivalent (QE) per g of dry extract. Total phenolic content were expressed as gallic acid (mg/g) using the following equation based on the calibration curve: y = 0.0054x+0.1321, where y is the absorbance and x is the concentration. Total flavonoid contents were expressed as quercetin (mg/g) using the following equation based on the calibration curve: y = 0.0064x+0.0763, where y was the absorbance and x is concentration.

Antioxidant activities

DPPH radical scavenging activity

The extract's antioxidant activity was measured using the DPPH free radical scavenging test, with slight modifications to the previously described method [19]. A total of 50 µL of the test sample at various concentrations was added to a solution containing 1.0 ml of 0.2 mmol DPPH. The mixture was then vortexed and left in the dark for 30 min. The solution absorbance was measured at a wavelength of 517 nm against a blank solution. The inhibition of each standard was expressed as % inhibition and calculated using the following equation (1).

$$\text{Scavenging rate } (\%) = [(\text{As} - \text{Ai})/\text{As}] \times 100\% \ldots\ldots\ldots (1)$$

Whereas represents the absorbance of DPPH without sample, and Ai represents the absorbance of the test sample mixed with the DPPH solution. Ascorbic acid was used as the reference standard. The experiment was conducted three times. The IC_{50} value was determined from a graph plotting the sample's concentration required to neutralize 50% of the DPPH free radicals.

$ABTS^+$ radical scavenging activity

The test procedure was performed with minor modifications in accordance with the previous described method [20]. ABTS powder (7.1 mg) and potassium persulfate powder (3.5 mg) were each dissolved in 5 ml of ethanol. The two solutions were then incubated in the dark for 12 h. After incubation, the solutions were mixed, and the final volume was adjusted to 25 ml with ethanol. MKE sample solutions at concentrations of 20, 40, 60, 80, and 100 mg/l were placed in test tubes, followed by the addition of the ABTS solution in a 1:1 ratio, and then homogenized. The absorbance of the mixture was measured at a wavelength of 520 nm. The free radical scavenging capacity was determined using equation (1).

Ferric ion reducing antioxidant potential (FRAP) test

The FRAP test was performed according to the previously described method [21]. The FRAP solution was prepared by mixing 10 ml of acetate buffer (0.3 M, pH 3.6), 1 ml of TPTZ (10 mmol, dissolved in 40 mmol hydrochloric acid), and 1 ml of ferric chloride (20 mmol). A 10 µl sample solution with varying concentrations was then mixed with 190 µl of FRAP solution and allowed to stand for 30 min at room temperature. Absorbance was measured at 700 nm, with the control (blank) solution containing all reagents except for the extract. Ascorbic acid was used as a positive control, and the IC_{50} value was calculated to express the results.

In vitro anti-inflammatory activities

The anti-inflammatory test was conducted using the bovine serum albumin (BSA) denaturation method based on Qamar *et al.,* with some modifications [22]. 0.45 ml of BSA was evenly mixed with MKE at various concentrations to form the reaction mixture (0.5 ml). After incubation at 40 °C for 25 min, 2.5 ml of PBS (pH 6.3) was added to the sample tube. The solution was then allowed to cool to room temperature, and absorbance was measured at 660 nm using a spectrophotometer. Sodium diclofenac was used as the standard, and PBS served as the control.

Data analysis

Mean±standard deviation (SD) was used to present the data. The data obtained were subjected to one-way analysis of variance (ANOVA). Statistical analysis was performed using SPSS software 2004.

The protein inhibition rate was calculated using the following equation (2):

$$\text{Inhibition}(\%) = (\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Treated}})/\text{Abs}_{\text{Control}} \times 100\% \ldots. (2)$$

The IC_{50} value can be calculated by creating a linear regression equation between concentration and percentage inhibition. If the inhibition percentage is>20%, the extract is considered to have anti-inflammatory activity [23].

RESULTS

Total phenolic and flavonoid contents

Antioxidant activity test using the DPPH method

The DPPH activity was measured by calculating the IC_{50} value using linear regression, which is the concentration of the test compound capable of reducing 50% of the DPPH radicals. In this study, MKE was tested at concentrations ranging from 20 to 50 mg/l. The results of the antioxidant activity test using the DPPH method are expressed as percentage inhibition (% inhibition) (table 2), which is then plotted against a series of sample or standard concentrations to generate a curve as shown in fig. 1.

Table 1: Yield, total amount of plant phenolic compound and flavonoids MKE

Sample	Yield (%)	[a]Total phenols mg/g plant extract (in GAE)	[b]Total flavonoids mg/g plant extract (in QA)
MKE	22.78	211.7329±1.10	157.2957±0.61

The GAE and QA values are expressed as mean±SEM of triplicate experiments. [a]Galic acid equivalents (GAE, mg/g of each extract) for the total phenolic content, [b]Quercetin equivalent (QA, mg/g of each extract) for the total flavonoid content.

Table 2: Antioxidant activity test result of the DPPH method

Sample	Concentration (mg/l)	Mean % Inhibition±SD	IC_{50}±SD
MKE	20	41.08±0.02	28.82 ±0.01
	25	44.43±0.02	
	30	53.49±0.07	
	40	61.62±0.07	
	50	72.27 ± 1.71	
Ascorbic Acid	2	43.80±0.08	3.78±0.01
	2.5	51.29±0.07	
	3	59.86±0.13	
	3.5	67.67±0.11	
	4	72.22±0.07	

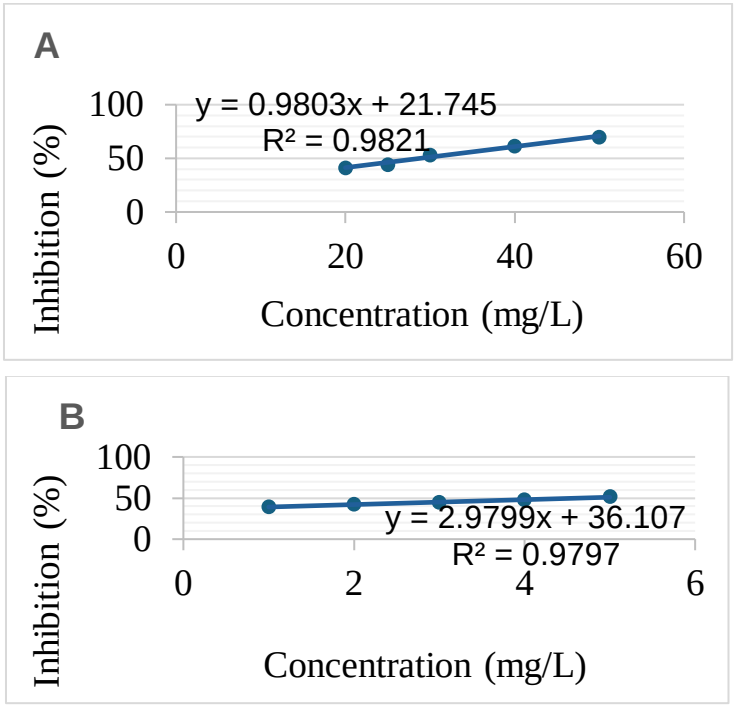

Fig. 1: Antioxidant activity curve of DPPH method (a) MKE, (b) ascorbic acid

Antioxidant activity test using the ABTS method

Based on the research conducted using the ABTS method, the IC_{50} value of MKE is expressed as percentage inhibition (% inhibition) (table 3), which is then plotted against a series of sample or standard concentrations to generate a curve as shown in fig. 2.

Antioxidant activity test using the FRAP method

Based on the research conducted using the FRAP method, the IC_{50} value of MKE is expressed as percentage inhibition (% inhibition) (table 4), which is then plotted against a series of sample or standard concentrations to generate a curve, as shown in fig. 3.

Inhibition of albumin denaturation

The IC_{50} value for protein denaturation inhibition is presented in table 5. Fig. 4 shows the percentage inhibition of albumin denaturation by MKE at various concentrations (5-40 µg/ml). Diclofenac sodium was used as the reference standard.

Table 3: Antioxidant activity test result ABTS method

Sample	Concentration (mg/l)	Mean % inhibition±SD	IC_{50}±SD
MKE	20	47.30±0.52	19.02±0.16
	25	55.80±0.76	
	30	61.97±0.22	
	40	66.67±1.40	
	50	72,27±1.71	
Ascorbic Acid	2	43,80±0.08	9.36±0.02
	2.5	51,29±0.07	
	3	59,86±0.13	
	3.5	67,67±0.11	
	4	72,22±0.07	

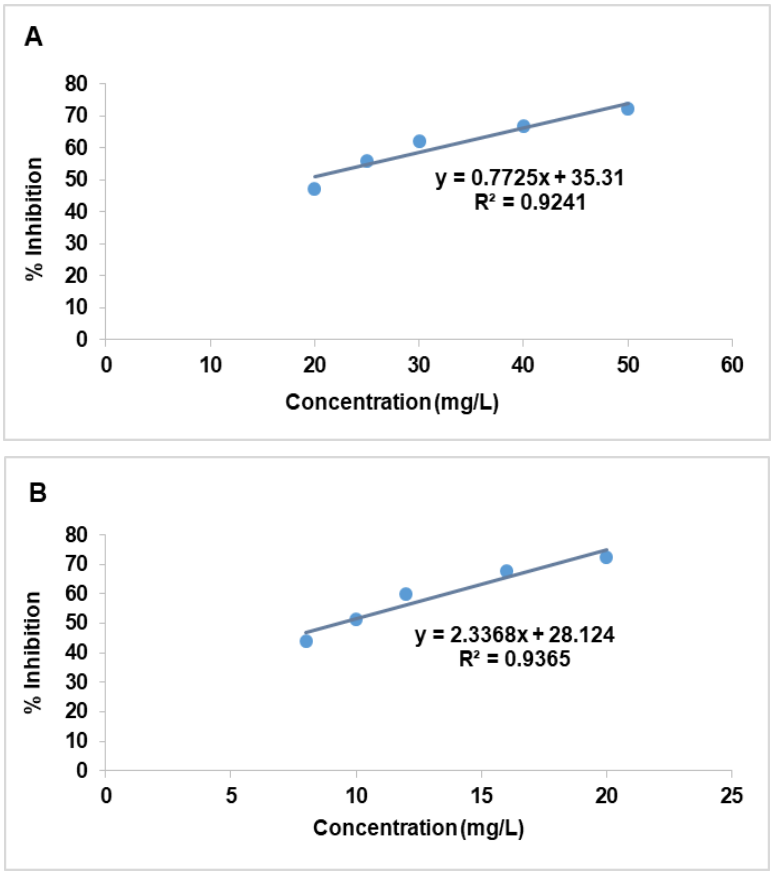

Fig. 2: Antioxidant activity curve of ABTS method (a) MKE (b) Ascorbic acid

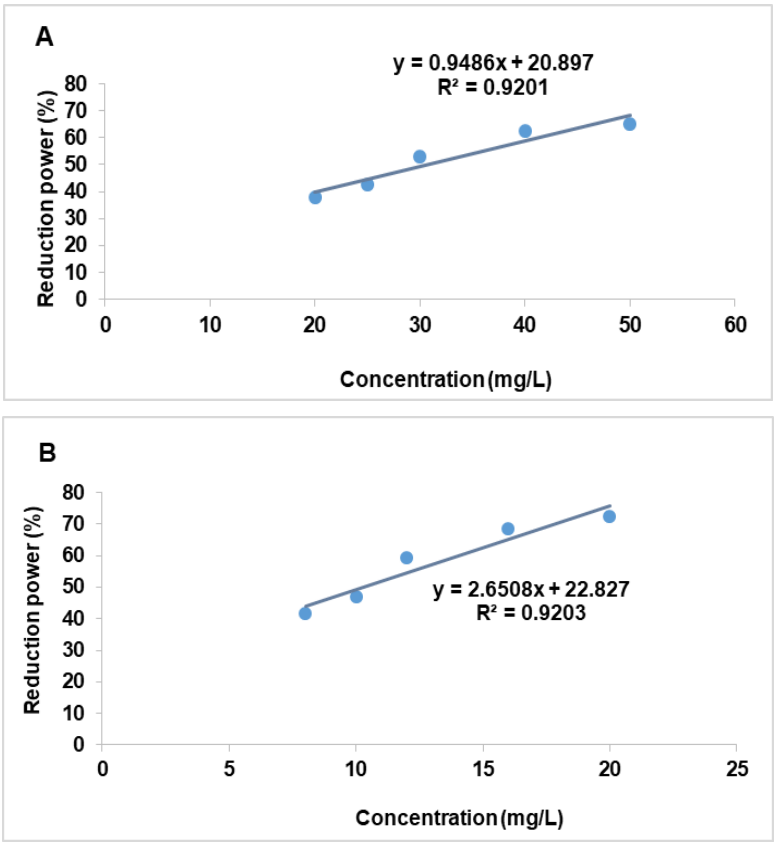

Fig. 3: Antioxidant activity curve of FRAP method (a) MKE, (b) ascorbic acid

Table 4: Antioxidant activity test result FRAP method

Sample	Concentration (mg/l)	Mean % inhibition±SD	IC_{50}±SD
MKE	20	37.94±0.04	30.68±0.11
	25	42.53±0.55	
	30	53.03±0.02	
	40	62.44±0.02	
	50	65.04±0.04	
Ascorbic Acid	8	41.83±0.15	10.25±0,01
	10	46.86±0.11	
	12	59.30±0.09	
	16	68.69±0.12	
	20	72.39±0.11	

Table 5: Test results of anti-inflammatory

Sample	Concentration (µg/ml)	Mean % inhibition±SD	IC_{50}±SD (µg/ml)
MKE	5	20.05±0.32	19.65±0.03
	10	29.60±0.57	
	20	47.10±0.48	
	30	75.58±0.83	
	40	91.29±0.78	
Diclofenac sodium	5	32.97±0.69	11.34±0.15
	10	49.54±0.40	
	20	70.73±0.80	
	30	85.07±0.37	
	40	92.54±0.37	

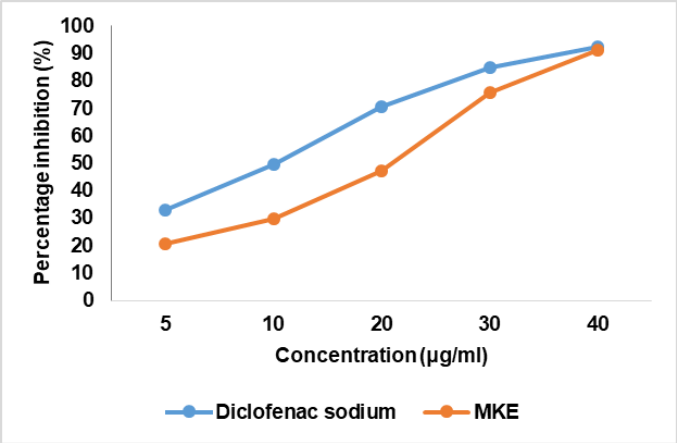

Fig. 4: Inhibition of protein denaturation of MKE and diclofenac sodium

DISCUSSION

Herbal plants have been used since ancient times to treat and manage various diseases, as they are considered a natural and healthy source of medicine [24]. The medicinal benefits of plants are derived from bioactive compounds that influence physiological activities in human body processes [25]. Various substances produced by plants, such as flavonoids and phenolic compounds, are known to have pharmacological activities that contribute to their therapeutic effects.

Table 1 shows the yield, phenolic content, and flavonoid content in MKE. The total phenolic content of MKE was determined using the Folin-Ciocalteu test, calculated from the regression equation y = 0.0054x+0.1321; R^2 = 0.9926, and expressed in gallic acid equivalents (GAE). Meanwhile, the flavonoid content in the extract was expressed in quercetin equivalents (QE) using the standard curve equation: y = 0.0064x+0.0763; R^2 = 0.9992. Phenols are able to modify the expression of genes encoding pro-inflammatory enzymes such as cytokines, lipoxygenase and cyclooxygenase; this, combined with their antioxidant potential, such as reactive oxygen species (ROS) scavenging, allows them to regulate inflammatory signaling. Their mechanism of action mainly involves the TNFα/NFκB signaling pathway [26].

Antioxidants are compounds that can slow down or prevent the oxidation process. These compounds can significantly inhibit or slow down the oxidation of easily oxidizable substances, even at low concentrations. DPPH is a stable free radical at room temperature and in methanol solvent, producing a purple-colored solution. When the DPPH free radical reacts with an antioxidant, its free radical properties are lost due to chain breaking, causing the solution color to change from purple to pale yellow [27].

The DPPH free radical is used to determine the radical scavenging effect of the leaf extract to evaluate antioxidant activity. As the concentration of MKE increases, the radical scavenging effect also increases, indicating that higher concentrations of MKE contain more metabolites that enhance the formation of DPPH-H bonds. As a result, DPPH changes to a yellow color, signifying lower absorbance and higher inhibition proportion [28]. Phenolic compounds in the extract, such as flavonoids, act as electron donors and donate

electrons to DPPH, leading to the color change from purple to yellow. This donation of electrons helps neutralize the free radical DPPH, reflecting the antioxidant potential of the extract [29]. The color change indicates the presence of antioxidant activity in the plant extract [30].

Based on the linear regression equation in fig. 1, the IC_{50} values for MKE and ascorbic acid were found to be 28.82±0.01 mg/l and 3.78±0.01 mg/l, respectively. In general, ascorbic acid demonstrated better DPPH radical scavenging activity compared to MKE. Referring to the standard antioxidant activity levels, a smaller IC_{50} value indicates a higher antioxidant activity, suggesting that ascorbic acid has a stronger ability to neutralize free radicals compared to MKE [31]. Antioxidant capacity is categorized as very strong (<50 ppm), strong (50–100 ppm), moderate (101–150 ppm), and weak (>150 ppm). Based on this classification, the antioxidant activity of MKE falls into the very strong category, as its IC_{50} value is<50 mg/l [32]. The antioxidant quality demonstrated by the samples mimics a number of studies previously conducted. *M. koenigii* leaves subjected to the ultrasonic-assisted extraction method reacted strongly with the DPPH assay (78.00±1.00%) followed by the microwave-assisted extraction (75.33±1.53%) and the solvent-assisted extraction (63.67±3.22%) [12].

The ABTS method can be used for both lipophilic and hydrophilic compounds, with a faster reaction time and the ability to operate within a wide pH range. However, this method is highly sensitive to light and requires a relatively long incubation period, typically 12–16 h in dark conditions [33]. The ABTS test relies on the formation of the blue/green ABTS+compound, which can be decolorized by antioxidant compounds. The principle of the ABTS method in measuring antioxidant activity is the reaction between the antioxidant compounds and the ABTS cation radical [30]. ABTS is a radical with a nitrogen-centered structure that exhibits a characteristic blue-green color, which will turn colorless when reduced by antioxidant compounds [34]. Before use, the ABTS reagent is reacted with potassium persulfate (K2S2O8) to form the ABTS• cation radical. This compound absorbs at a wavelength of 743 nm, showing a bluish-green color, and will decolorize when receiving an electron or hydrogen atom donation from the antioxidant compound [35]. Based on the regression equation obtained in fig. 2, the IC_{50} values for ascorbic acid and MKE are 9.36±0.02 and 19.02±0.16 mg/l, respectively. Based on categorization, the antioxidant activity of MKE using the ABTS method falls into the very strong category because its IC_{50} value is<50 mg/l [32].

The FRAP method has advantages such as fast results, ease of use, and the ability to identify antioxidants in complex matrices [36]. Based on the regression equation in fig. 3, the IC_{50} values for MKE and ascorbic acid are 30.68±0.11 mg/l and 10.25±0.01 mg/l, respectively. Based on categorization, the antioxidant activity of MKE using the FRAP method falls into the very strong category because its IC_{50} value is<50 mg/l [32].

Severe oxidative stress can impair the activity of several enzymes, leading to the development of various diseases such as inflammation, diabetes, cancer, heart disease, rheumatoid arthritis, and others. Therefore, the presence of antioxidant properties in a plant increases the likelihood of it having anti-inflammatory, anti-arthritis, and other beneficial effects [37]. The anti-inflammatory activity of *Murraya koenigii* extract (MKE) was assessed using protein denaturation.

Protein denaturation has been identified as one of the primary causes of inflammation. During protein denaturation, the secondary and tertiary structures of proteins are lost due to external stress, heat, organic solvents, or strong acids or bases. The denaturation mechanism involves changes in electrostatic, hydrogen, hydrophobic, and disulfide bonds [38]. Additionally, complex enzyme activation, the release of inflammatory mediators, cell migration, as well as tissue degradation and repair processes occur, leading to the loss of molecular conformation and protein denaturation. Therefore, compounds capable of preventing these changes and inhibiting protein denaturation caused by heat or thermal stress have potential therapeutic value as anti-inflammatory agents.

The inhibition of albumin denaturation by MKE is presented in table 3. The percentage of inhibition indicates that MKE exhibits significant anti-inflammatory activity by preventing the denaturation of bovine serum albumin. A study has reported that compounds capable of inhibiting protein denaturation by more than 20% are considered to have anti-inflammatory activity and can be further developed [39].

The linear correlation analysis demonstrates a strong relationship between concentration and percentage inhibition (fig. 4). The half-inhibitory concentration (IC_{50}) of MKE and diclofenac sodium is 19.65 µg/ml and 11.39 µg/ml, respectively. These results indicate that MKE has potential as an anti-inflammatory agent. Phenolic compounds function similarly to non-steroidal anti-inflammatory drugs (NSAIDs) by inhibiting inflammatory mediators. Additionally, phenolic compounds can suppress cyclooxygenase enzymes and the NF-κB signaling pathway [40]. Flavonoids have been reported to inhibit cyclooxygenase and lipoxygenase enzymes, thereby reducing the synthesis of inflammatory mediators such as prostaglandins, leukotrienes, histamine, bradykinin, and thromboxane [41].

CONCLUSION

MKE demonstrates strong antioxidant capacity and significant anti-inflammatory potential, making it a promising herbal source. Although this study demonstrates the significant anti-inflammatory and antioxidant potential of *M. koenigii*, several limitations should be considered. Variability in plant material quality, differences in extraction methods, and potential interactions between bioactive compounds may affect the consistency and reproducibility of the results. Additionally, *in vivo* and clinical studies are needed to determine the optimal dosage, long-term safety, and potential interactions with other treatments. Further research is also required to isolate and characterize the major active compounds and to gain a deeper understanding of their mechanisms of action.

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CONFLICTS OF INTEREST

All authors have none to declare

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