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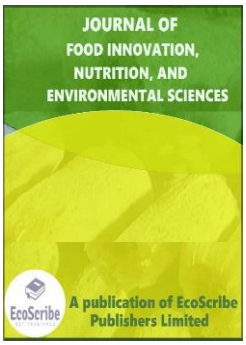

ORIGINAL ARTICLE

Influence of Leaf Physical Form and Heating on the Antioxidant Potential of Curry Leaves (*Murraya koenigii*)

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ABSTRACT

The study evaluated the effect of leaf physical form and heating on antioxidant activity, total phenolic content and flavonoid content of *Murraya koenigii*. Aqueous extracts were prepared from compound leaves and leaflets in two forms: powder and whole leaves. Antioxidant activity (IC_{50}) was assessed using the DPPH (2,2-Diphenyl-1-Picrylhydrazyl) assay, while total phenolic and flavonoid contents were measured by Folin-Ciocalteu and aluminium chloride colorimetric methods respectively. Antioxidant activity, phenolic content, and flavonoid content of *Murraya koenigii* extracts varied significantly with leaf form and heating duration. Powdered compound leaves and powdered leaflets exhibited the strongest antioxidant activity, with an IC_{50} of 0.0679 and 0.0764 mg/ml, respectively, while whole leaves demonstrated significantly weaker activity. Heating time significantly influenced antioxidant activity ($P < 0.05$). with an overall trend of improved activity at longer durations (15-60 minutes). However, the actual values at shorter durations (5-10 minutes) particularly in powdered forms, also showed strong activity, indicating that antioxidant levels did not consistently increase with prolonged heating. The highest phenolic content was recorded in powdered compound leaves at 45 minutes (43.66 ± 1.03 mg GAE/g), while the average across all treatments at this time point was 34.19 mg GAE/g. Prolonged heating significantly enhanced total phenolic content across all leaf forms ($P < 0.05$). The highest flavonoid content was in powdered leaflets at 5 minutes (36.21 ± 0.33 mg QE/g), with an average peak of 33.13 mg QE/g at the 45 minutes. Flavonoid levels also increased significantly with heating across all leaf forms ($P<0.05$). A highly significant interaction ($P < 0.001$) between leaf form and heating duration was observed, indicating the combined impact on *Murraya koenigii*'s antioxidant potential.

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1. INTRODUCTION

Over the last few decades, it has become apparent that most degenerative diseases are associated with reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion ($O2^{\bullet-}$), and the hydroxyl radical ($OH^{\bullet}$) (Afzal *et al.,* 2023). ROS arise in various ways including cellular metabolic reactions, exposure to UV light, ionizing radiation, or heavy metal ions (Nita & Grzybowski 2016). Under stress conditions, the body produces increased levels of ROS leading to homeostatic imbalance and oxidative stress. This result in lipid peroxidation, proteins oxidation, nucleic acid damage, enzyme inhibition, activation of cell death, and tissue damage (Juan *et al.,* 2021). ROS accumulation promotes the development of age-dependent diseases, such as cancer, atherosclerosis, arthritis, etc. (Nita and Grzybowski 2016; Sharma *et al.*, 2011). Antioxidants play an important role in biological systems by suppressing the formation of ROS (Jomova 2023). There are endogenous and exogenous systems to protect cells from damage in animals. However, in many conditions, the endogenous antioxidants are depleted and therefore the exogenous supply of antioxidants becomes essential. Since synthetic antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), n-propyl gallate (PG) pose a potential health risk due to contamination with chemical precursors, toxic solvents, and the formation of hazardous by-products, natural antioxidants are an attractive alternative (Flieger *et al.,* 2021; Xu *et al.,* 2021). The sources of natural antioxidants are mainly plants, which are rich in vitamins, phenolic compounds, and carotenoids (Lü *et al.,* 2010; Anwar *et al* 2018; Amarowicz & Pegg 2020).

Murraya koenigii L. is commonly known as a curry plant (Karapincha; vernacular name in Sinhala) and belongs to the family Rutaceae. The plant is native to Sri Lanka, and other South Asian countries and is cultivated throughout Sri Lankan gardens. *Murraya koenigii* is important due to its broad range of traditional medicinal properties. Curry leaves are highly valued in many food products due to their flavour, aroma, and medicinal importance. Leaves are mostly used as a flavouring agent in cooking Sri Lankan vegetables, meat, and fish dishes. Further sambal made from fresh ground curry leaves and porridge is also famous for its nutritional value. Various studies have reported that the compounds isolated from the leaves of *M. koenigii* namely mahanimbine, isomahanine, isolongifolene, 9-formyl-3-methyl carbazole, koenine, koenigine, O-methylmurrayamine, muconicine, murrayacinine, mahanimbolin, mukoeic acid, urrayanine, have exhibited antioxidant properties (Jagan *et al.,* 2007; Gupta *et al*., 2009; Balakrishnan *et al.,* 2020). Fresh or dried leaflets or compound leaves are often used in cooking and in some instances, leaves are used in dried powder form. This study compared the levels of antioxidants extracted into an aqueous solution from leaflets and compound leaves, evaluating both powdered and whole-leaf forms.

2. MATERIALS AND METHODS

2.1 Sample preparation and extraction

In this study compound leaves and leaflets of curry leaves (*Murraya koenigii)* were used in the powdered form and as whole leaves. Samples were collected from a home garden in the Kandy area of Sri Lanka and were authenticated by comparison with the herbarium specimens at the National Herbarium, Department of National Botanic Gardens, Peradeniya, Sri Lanka

Clean, healthy, mature leaves were picked at about 4-5 compound leaves below the bud. The whole compound leaves (WC) and leaflets (WL) were collected separately and air-dried at room temperature without exposure to sunlight until a constant weight remained, and stored -20ºC until further analysis. A part of the dried leaflets and compound leaves were ground separately to a fine powder using a domestic electric mixer grinder (Panasonic MX-216E). The powdered leaflets (PL) and powdered compound leaves (PC) were stored at -20ºC until further analysis.

The sample mixture for the extraction was prepared by combining 1g of each form (WC, WL, PC, PL) with 10 ml of distilled water in screw-capped test tubes. Seven tubes for each sample were prepared (7 x 4 for WC, WL, PC, and PL each). Then the samples were kept in a water bath at 100ºC and one sample from each of the leaf forms was removed after 0, 5, 10, 15, 30, 45, and 60 minutes. The resultant suspension was centrifuged at 10,000 × g for 10 min at 4 °C and filtered through Whatman No. I filter paper and was lyophilized (Telstar-Lyo-Quest, Japan) and stored at –20°C for further studies.

A dilution series of 1.0, 0.5, 0.25, 0.125, 0.063, 0.032, and 0.016 mg/mL was prepared from each of the lyophilized aqueous extracts of PL, PC, WL, and WC, obtained after heat treatment at 0, 5, 10, 15, 30, 45, and 60 minutes, by re-dissolving in 30% methanol, a solvent commonly used to enhance solubility and compatibility in radical scavenging and phenolic content assays.

2.2 Determination of free radical scavenging activity of aqueous extract by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

DPPH assay for total antioxidant capacity was performed as described by Wanigasekera *et al.,* (2019). The reaction mixture was prepared with 1.96 ml of 8 x10^{-5} M methanolic solution of DPPH and 40 µl of plant extract at concentrations ranging from 0.016 to 1 mg/ml. Ascorbic acid was used as a reference standard. All samples were incubated at room temperature for 20 minutes in the dark and then the absorbance was measured at 517 nm using the PG T80 plus UV-visible spectrophotometer. Measurements were performed in triplicates. The percentage of DPPH radical scavenging activity was determined using the formula:

% Inhibition = $[(A_{DPPH} - A_{Sample})/A_{DPPH}] \times 100$,

where $A_{(DPPH)}$ was the absorbance of DPPH control solution and $A_{(Sample)}$ was the absorbance of DPPH solution in the presence of plant extract. Sample concentration giving 50% inhibition was estimated as IC_{50} value using the dose inhibition curve in linear range by plotting the extract concentration versus the corresponding scavenging activity.

2.3 Determination of Total Phenolic Content

The total phenolic content (TPC) of each of the treated leaves of *Murraya koenigii* was determined using Folin-Ciocaltue reagent method with slight modifications. (Singleton *et al.,*1999; Wanigasekera *et al.,* 2019). An aliquot (20 µl) of gallic acid calibration standards (50 mg/ml to 1000 mg/ml) was mixed with 1.58 ml of distilled water and 100 µl of Folin-Ciocalteu's reagent. The reaction mixture was incubated at room temperature for 8 min and then 300 µl of 200 mg/ml sodium carbonate was added, re-incubated for another 2 hours at room temperature and absorbance was measured at 765 nm. Analysis was performed in triplicate and the average absorbance was used to plot the calibration graph. The same procedure was repeated for the plant extracts. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration graph and the content of phenols was expressed in terms of gallic acid equivalents per gram of extract (mg of GAE/g). Data are expressed as mean ± SD of three replicates.

2.4 Determination of Flavonoid content by Aluminium chloride colorimetric assay

Total flavonoid content was determined using the Aluminum chloride colorimetric method (Pereira *et al.,* 2012; Thangaraj 2016; Wanigasekera *et al.,* 2019). Quercetin (QE) calibration standards of 0.04 mg/ml to 0.5 mg/ml (500µl) were pipetted into 5ml volumetric flasks with 2ml of distilled water and 0.15 ml of 5% $NaNO_2$. The mixture was incubated at room temperature for 5 minutes. Then, 0.15 ml of 10 % $AlCl_3$ was added to each sample tube, except for the blank to which the same volume of distilled water was added. The samples were incubated again at room temperature for 6 minutes, and 1 ml of 4% NaOH was added, vortexed, and brought to a final volume of 5 ml with distilled water. Absorbance was measured at 430 nm after 15 minutes. All dilutions were analyzed in triplicate and average absorbance was used to plot the calibration graph. The standard graph was drawn with absorbance against the concentration of QE dilutions. Data are expressed as mean ± SD of three replicates. The same procedure was repeated with the plant extract and the content of the flavonoids was expressed in terms of QE equivalents per gram of plant extract.

2.5 Statistical analysis

A two-way analysis of variance (ANOVA) was performed to evaluate the effects of leaf form and heating time on DPPH free radical scavenging activity, total phenolic content, and total flavonoid content. Tukey's post-hoc test was used to determine significant differences between treatment means at a significant level of $P<0.05$. Significant interactions between leaf form and heating time were assessed, with results considered statistically significant at $P<0.05$, $P<0.01$, and $P<0.001$, denoted by *, **, and ***, respectively. Statistical analyses were performed using R statistical software R Core Team (2023).

3. RESULTS AND DISCUSSION

3.1 Antioxidant activity (DPPH Assay)

DPPH analysis is widely accepted method for evaluating antioxidant capacity in plant extracts (Pérez- Jiménez *et al.,* 2008). DPPH is a stable free radical characterized by a deep violet colour, which becomes pale yellow upon reaction by an antioxidant (Kedare and Singh, 2011). In this study, the antioxidant potential of *Murraya koenigii* extracts was evaluated using the DPPH assay, and the IC_{50} values (mg/ml) were calculated from dose response curves. The results are presented in **Table 1.**

The DPPH free radical scavenging activity (IC_{50} values) of different *Murraya koenigii* extracts showed significant differences based on both leaf form and heating duration ($P < 0.05$) **(Table 2).** Among the different leaf forms, powdered compound leaves ($IC_{50} = 0.0679^{d}$ mg/ml) and powdered leaflets ($IC_{50} = 0.0764^{e}$ mg/ml) exhibited the strongest antioxidant activity. In contrast, whole compound leaves (WC) and whole leaflets (WL) showed significantly weaker antioxidant activity ($IC_{50} = 0.1511^{b}$ and 0.1745^{a} mg/ml, respectively), likely due to limited surface area and inefficient solvent penetration, resulting in reduced extraction of bioactive compounds (Sharma *et al.,* 2018).

Similarly, heating duration also had a significant impact on antioxidant activity ($p < 0.05$). The lowest average IC_{50} value across all forms was at 15 minutes (0.0957^{e} mg/ml**),** followed closely by samples heated for 30, 45, and 60 minutes. This suggests that heating up to 15–60 minutes generally enhances antioxidant activity, though the peak effect was observed at 15 minutes. However, the actual lowest IC_{50} (i.e., highest antioxidant activity) was observed in powdered compound leaves at 5 minutes (0.052 ± 0.001 mg/ml)**.** This suggests that short-duration heating of finely ground leaf material may be particularly effective**,** possibly due to rapid release of thermally stable phenolic compounds and enhanced solubility (Xu *et al*., 2017). Importantly, the interaction between leaf form (A) and heating time (B) was highly significant ($P < 0.001$), indicating a combined effect on DPPH radical scavenging capacity.

Table 1. Effect of leaf form and heating time on antioxidant, phenolic, and flavonoid contents of *Murraya koenigii* extracts

Leaf Form	Time (min)	DPPH IC_{50} (mg/ml)	Total Phenolic (mg GAE/g)	Total Flavonoid (mg QE/g)
Powdered Compound (PC)	0	0.077 ± 0.001	27.40 ± 0.32	25.94 ± 0.11
	5	0.052 ± 0.001	32.35 ± 0.23	27.72 ± 0.24
	10	0.053 ± 0.002	26.29 ± 2.21	24.68 ± 0.15
	15	0.069 ± 0.001	25.53 ± 1.67	24.57 ± 0.08
	30	0.074 ± 0.002	34.56 ± 1.55	30.29 ± 0.05
	45	0.070 ± 0.001	43.66 ± 1.03	35.99 ± 0.42
	60	0.081 ± 0.005	40.25 ± 0.96	33.06 ± 0.16
Powdered Leaflets (PL)	0	0.053 ± 0.001	26.11 ± 0.96	29.71 ± 0.19
	5	0.057 ± 0.005	35.73 ± 0.52	36.21 ± 0.32
	10	0.056 ± 0.002	32.59 ± 1.36	35.08 ± 0.07
	15	0.073 ± 0.001	26.77 ± 2.27	30.55 ± 0.06
	30	0.083 ± 0.001	24.23 ± 2.31	28.35 ± 0.07
	45	0.091 ± 0.001	34.89 ± 0.65	35.79 ± 0.07
	60	0.121 ± 0.002	31.40 ± 0.44	31.47 ± 0.03
Whole Compound (WC)	0	0.240 ± 0.003	17.55 ± 0.21	21.79 ± 0.22
	5	0.157 ± 0.007	22.67 ± 1.83	25.27 ± 0.59
	10	0.164 ± 0.001	20.55 ± 0.38	23.72 ± 0.25
	15	0.136 ± 0.001	28.54 ± 1.59	25.93 ± 0.76
	30	0.148 ± 0.003	24.40 ± 1.19	29.88 ± 0.15
	45	0.114 ± 0.000	28.08 ± 2.30	28.97 ± 0.32
	60	0.098 ± 0.001	32.28 ± 0.18	30.77 ± 0.24
Whole Leaflets (WL)	0	0.194 ± 0.012	17.11 ± 1.16	22.42 ± 0.24
	5	0.445 ± 0.006	11.90 ± 0.61	18.77 ± 0.23
	10	0.152 ± 0.002	21.38 ± 0.40	23.59 ± 0.49
	15	0.104 ± 0.002	26.01 ± 0.78	25.55 ± 0.82
	30	0.103 ± 0.002	25.86 ± 1.23	27.80 ± 0.39
	45	0.120 ± 0.001	30.15 ± 0.58	31.78 ± 0.35
	60	0.104 ± 0.002	29.56 ± 2.14	33.20 ± 0.30

Notes: Values are expressed as mean ± SD (n = 3). IC_{50} = concentration required to inhibit 50% of DPPH radicals. GAE = gallic acid equivalent. QE = quercetin equivalent.

3.2 Total Phenolic Content

Phenolic compounds are key bioactive constituents in plants, widely recognized for their antioxidant potential, which primarily arises from their redox properties and ability to donate hydrogen atoms or electrons. These compounds play an essential role in neutralizing free radicals and protecting cells from oxidative damage. In *Murraya koenigii,* total phenolic content (TPC) was quantified using the Folin Ciocaltue colorimetric method, a widely accepted assay for measuring phenolics in plant extracts (Pérez *et al.,* 2023). The assay is based on the formation of a blue-colored complex upon reaction with phenolics, which is measured spectrophotometrically. A calibration curve was prepared using standard gallic acid solutions, resulting in the calibration equation y = 0.8243x + 0.0135, confirming a strong linear correlation between absorbance and phenolic concentration (**Figure 1**). TPC values were expressed in mg of gallic acid equivalents (GAE) per gram of dry extract. The mean ± SD values of TPC across different heating durations and leaf forms are given in **Table 1**. The highest phenolic content was observed in powdered compound leaves (PC) heated for 45 minutes (43.66 ± 1.03 mg GAE/g), followed by PC at 60 minutes (40.25 ± 0.96 mg GAE/g) and powdered leaflets (PL) at 5 minutes (35.73 ± 0.52 mg GAE/g) (**Table. 1**) Overall, powdered samples (PC and PL) consistently exhibited higher TPC than whole leaf forms, likely due to increased surface area, which facilitates solvent penetration and the release of bound phenolics. As shown in **Table 2**, statistical analysis confirmed significant differences ($P < 0.05$) in TPC based on both leaf form and heating time.

Powdered compound leaves exhibited the highest phenolic content (32.86^{a} mg GAE/g), followed by powdered leaflets (30.24^{b} mg GAE/g), while whole compound leaves (24.86^{c}) mg GAE/g, and whole leaflets (23.13^{d} mg GAE/g) showed comparatively lower values. Heating time also significantly

influenced phenolic content, with the highest average values observed at 45 minutes (34.19[a] mg GAE/g) and 60 minutes (33.37[a] mg GAE/g) ($P < 0.05$) indicating that moderate thermal treatment enhances phenolic extraction. Furthermore, a highly significant interaction between leaf form and heating duration was observed ($P < 0.001$), indicating that the impact of heat on phenolic release was highly dependent on leaf structure. These findings suggest that both physical form and heat treatment play a critical role in optimizing phenolic yield in *Murraya koenigii* extracts.

Table 2. DPPH free radical scavenging activity (IC_{50}), Total Phenolic Content, and Total Flavonoid Content in *Murraya koenigii* extracts based on leaf form, heating time, and their interaction

Factor	DPPH IC_{50} (mg/ml)	Phenolic Compounds (mg GAE/g extract)	Flavonoid Compounds (mg QE/g extract)
Leaflets	0.1745[a]	23.13[d]	26.15[d]
Compound leaves	0.1511[b]	24.86[c]	26.62[c]
Powdered leaflets	0.0764[c]	30.24[b]	32.45[a]
Powdered compound leaves	0.0679[d]	32.86[a]	28.89[b]
Heating Time (min)			
0	0.1412[b]	22.04[d]	24.96[e]
5	0.1774[a]	25.66[bc]	26.99[d]
10	0.1063[c]	25.20[c]	26.76[d]
15	0.0957[e]	26.71[bc]	26.64[d]
30	0.1020[d]	27.26[b]	29.08[c]
45	0.0987[de]	34.19[a]	33.13[a]
60	0.1012[d]	33.37[a]	32.12[b]
Source of Variation	DPPH IC_{50}	Phenolic	Flavonoid
Leaf Form (A)	***	***	***
Heating Time (B)	***	***	***
Interaction (A × B)	***	***	***

Note: Different superscript letters within each column indicate significant differences (Tukey's HSD test, $P < 0.05$). *, **, and *** indicate significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively

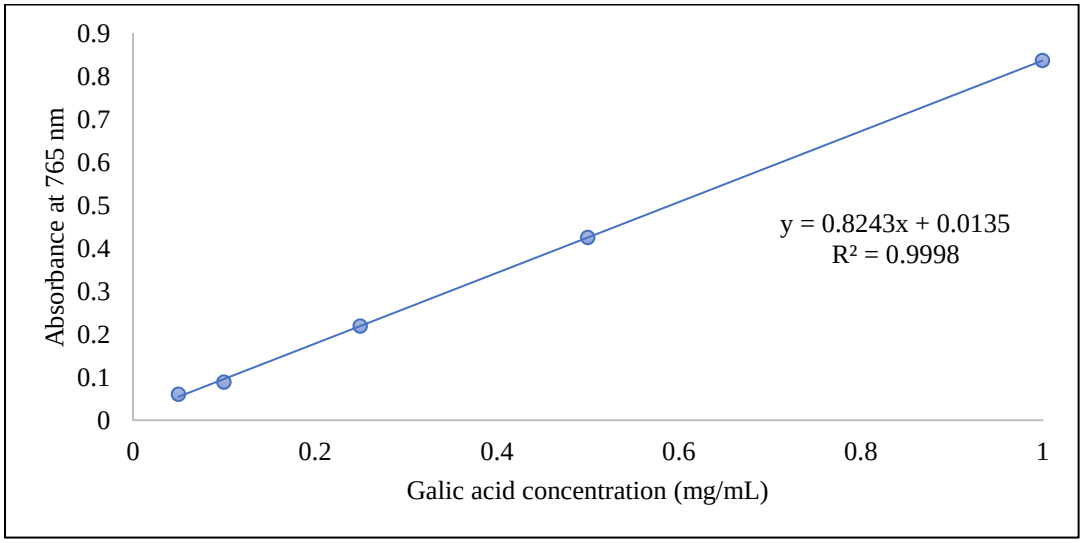

Fig. 1. Standard gallic acid calibration graph for Phenolic content.

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3.3 Total Flavonoid Content

The total flavonoid content of *Murraya koenigii* extracts was quantified using the aluminum chloride ($AlCl_3$) colorimetric method, a validated technique for flavonoid estimation (Thangaraja, 2016). This method involves the formation of a yellow complex between flavonoids and $AlCl_3$, which absorbs at 430 nm. The intensity of this color, measured spectrophotometrically, correlates directly with flavonoid concentration. A standard curve was constructed using quercetin solutions, yielding the equation $y = 1.112x + 0.0164$ ($R^2 = 0.99$), confirming a strong linear relationship between absorbance and flavonoid content (**Figure 2**).

Table 1 presents the mean ± SD values of flavonoid content in extracts calculated as quercetin equivalents (mg QE). Based on actual data, the highest flavonoid content was observed in powdered leaflets (PL) at 5 minutes (36.21 ± 0.32 mg QE/g), followed by PL at 10 and 45 minutes (35.08 ± 0.07 and 35.79 ± 0.07 mg QE/g, respectively). Powdered compound leaves (PC) also demonstrated high flavonoid levels, peaking at 45 minutes (35.99 ± 0.42 mg QE/g). Statistically, the average flavonoid content was significantly higher ($P < 0.05$) in powdered leaflets (32.45 mg QE/g), followed by powdered compound leaves (28.89 mg QE/g), whole compound leaves (26.62 mg QE/g), and whole leaflets (26.15 mg QE/g) (**Table 2**) In terms of heating time, 45 minutes resulted in the highest flavonoid content (33.13 mg QE/g), across all forms while the lowest was observed at 0 minutes (24.96 mg QE/g). These findings indicate that both physical processing and thermal treatment enhance flavonoid extraction, with optimal values occurring in powdered forms. A highly significant interaction between leaf form and heating time ($P<0.001$) was observed, indicating that the combination of these factors strongly influenced flavonoid levels.

The antioxidant properties of *Murraya koenigii* are primarily attributed to its phenolic compounds, flavonoids, and alkaloids (Balakrishnan *et al.,* 2020; Abeysinghe *et al.,* 2021; Rajendran *et al*., 2014). Numerous studies have documented the antioxidant activity of *Murraya koenigii,* using various extraction solvents and methods (Tomar *et al*., 2018; Aju*, et al* 2017). In the present study, water was used as the extraction medium, considering its relevance to dietary use particularly in South and Southeast Asian cuisines where curry leaves are commonly consumed. Our finding demonstrates that powdered *Murraya koenigii* leaves, when subjected to heating durations between 15 to 60 minutes, exhibit the highest antioxidant properties along with the greatest phenolic and flavonoid contents. The powdered form enhanced the extraction efficiency due to its increased surface area, which facilitates better solvent penetration and release of bioactive compounds. Additionally, heating up to 60 minutes improved compound release without clear evident of thermal degradation, indicating that this temperature range is suitable for maximizing bioactive yield while maintaining stability. These results are consistent with existing literature, which suggests that physical processing methods such as powdering and controlled heating can significantly enhance the extraction and stability of bioactive compounds in plant materials (Jha and Sit 2022; Lobo *et al.,* 2010; Pandey & Rizvi, 2009). Together, these results underscore the importance of selecting appropriate extraction conditions to optimize the functional properties of *M. koenigii*.

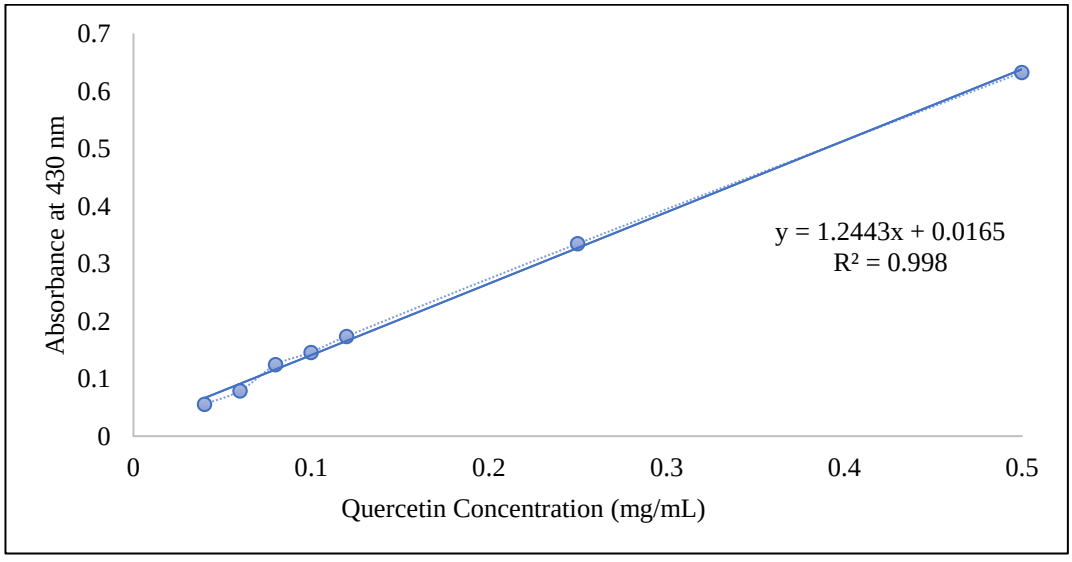

Fig. 2. Standard Quercetin calibration graph for Flavonoid content

CONCLUSION

The antioxidant potential, phenolic content, and flavonoid levels in *Murraya koenigii* are significantly influenced by both the physical form of the leaves and the duration of heating. Powdered leaves enhanced the extraction of bioactive compounds compared to the whole leaf forms. Based on statistical analysis, antioxidant activity (as indicated by IC_{50} values) generally improved with heating durations between 15 and 60 minutes. However, individual results revealed that certain powdered forms, particularly at 5–10 minutes, exhibited the strongest antioxidant activity as reflected by the lowest IC_{50} values. Shorter heating times appear favorable for maximizing antioxidant capacity, while prolonged heating enhances yield of phenolic and flavonoid compounds. Therefore, this study highlights the importance of selecting appropriate processing conditions to optimize the functional properties of *Murraya koenigii* for dietary and therapeutic applications.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this work.

DATA AVAILABILITY

The data used to support the findings of this study are available upon request from the corresponding author.

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