

Full Length Research Paper

Reverse phase-high performance liquid chromatography-diode array detector (RP-HPLC-DAD) analysis of flavonoids profile from curry leaf (*Murraya koenigii*. L)

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Murraya koenigii L. Spreng, a medicinally important herb of Indian origin, has been used for centuries in the Ayurvedic system of traditional Indian medicine and popularly used in Indian cuisine on a daily basis. To evaluate the quality of *M. koenigii*, L. leaves, a sensitive, simple and precise reversed-phase high performance liquid chromatography (RP-HPLC) method was developed for assessment of four major bioactive flavonoids: rutin, quercetin, myricetin and kaempferol. Separation was achieved on a reversed phase column (ZORBAX, Eclipse plus-C₁₈, 5 µm, 4.6 × 150 mm, Agilent, USA) and methanol:acetonitrile:water:acetic acid (40:20:39:1, v/v/v/v) was employed as the eluent. Sample was eluted at 0.8 ml/min and peaks were simultaneously identified using UV absorbance at 350 nm for kaempferol and 254 nm for rutin, myricetin, and quercetin. The authenticated four analytes were used to construct linear standard curves by injecting range of 20 to 200 ng. The correlation coefficients of the calibration curve for the analytes existed higher than 0.999. Isolation of four compounds in *M. koenigii*, leaves was achieved by the RP-HPLC method and the results showed that mean value of rutin (924.25 mg/kg) and quercetin (85.88 mg/kg) accumulated greater concentration. Lowest flavonoid concentration 5.88 and 0.20 mg/kg were found to possess in myricetin and kaempferol, respectively. Total flavonoids concentration was observed to be 1415.50 mg/kg. This study suggested that accumulation of the greater amount of bioactive components: rutin and quercetin in *M. koenigii* leaves will be more useful information for further pharmacological investigations.

Key words: Flavonols, polyphenol, rutin, *Murraya koenigii*, reversed-phase high performance liquid chromatography (RP-HPLC).

INTRODUCTION

Flavonoids belong to a group of natural substances with variable phenolic structures and abundance in fruits,

vegetables, grains, bark, roots, stems, flowers, tea, wine and medicinal plants (Pekkarinen et al., 1999). Flavonoids

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are the most ubiquitous groups of plant phenolics. Due to their importance in food organoleptic properties and human health, a better understanding of their structures and biological activities indicates their potentials as therapeutic agents and also for predicting and controlling food quality (Tapas et al., 2008). Researchers have attracted the greatest attention, and have been extensively studied, because they are highly effective antioxidants with lower toxicity than synthetic antioxidants such as butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT) (Pekkarinen et al., 1999).

Every group of flavonoids has their capacity to act potential as antioxidants. Among them, flavones and catechin components predominately acted as the most powerful flavonoids in protecting the body against reactive oxidative species damage (De Groot, 1994). Several studies reported that quercetin had the ability to inhibit low-density lipoproteins (LDL) oxidation (Graf et al., 2005), and the oxidation of LDL can result in the formation of atherosclerotic plaques, leading to cardiovascular disease (Hollman and Katan, 1997). Quercetin, acting as free radical scavengers was shown to exert a protective effect in liver reperfusion ischemic tissue damage. In addition, quercetin can also reduce inflammation by scavenging free radicals. Free radicals can activate the transcription factors that generate pro-inflammatory cytokines, which are frequently found, elevated in patients that suffer from chronic inflammatory diseases (Boots et al., 2008). Quercetin, rutin, myricetin, and kaempferol, acted as antioxidants, exhibited beneficial effects such as anti-inflammatory, antiviral, and antiallergic, in addition to anticancer activity (Hillwell, 1994; Fraga et al., 1987).

Curry leaf *Murraya koenigii* (Rutaceae), is a perennial plant commonly known as Curry Veppilai (Tamil) and "Curry Patta" (Hindi) is widely used as a spice throughout India and other tropical countries (Joseph and Peter, 1985). *M. koenigii*, a medicinally important herb of Indian origin, has been used for centuries in the Ayurvedic system of medicine, and very popularly used in Indian cuisine on daily basis. Several parts of *M. koenigii* have been used in traditional medicine for the treatment of rheumatism, traumatic injury and snake bite, because of it has been reported to have antioxidant (Ninjappa et al., 2008), antimicrobial (Goutam and Purohit, 1974), anti-diabetic and anti-dysenteric activities (Kong et al., 1986; Kesari et al., 2007; Mishra et al., 2009; Yankuzo et al., 2011). It has also been used as anti-inflammatory (Muthumani et al., 2009), anticancer (Kok et al., 2012; Muthumani et al., 2009), antinociceptive (Patil et al., 2012), anthelmintic (Sharma et al., 2010), anticholinesterase and anti-amnesic activity (Tembhurne and Sakarkar, 2011). The recent research reported curry leaves to have a rich source of polyphenols, inhibit the proteolytic activity of the cancer cell proteasome, and cause cell death (Noolu et al., 2013).

Earlier studies of *M. koenigii*, the phytochemical analysis was done mostly using thin layer chromatography (TLC), HP-TLC, and Spectrophotometer. The detailed data for the extraction of the individual flavonoids from *M. koenigii* leaves were limited, and until none of them was characterized by all the components. Therefore, the present study was carried out to find appropriate precise extraction method and quantification of flavonoids from *M. koenigii* leaves extracts using reversed phase-high performance liquid chromatography-diode array detector (RP-HPLC-DAD) analysis. In addition, validate the leaf flavonoid components, thus to obtain an informative profile which may serve as a basis for further utilization of *M. koenigii* leaves. Furthermore, the results will be important as an indication of *M. koenigii* leaves flavonoids as a new source of bioactive flavonoids.

MATERIALS AND METHODS

Plant

Freshly harvested *M. koenigii* L. Spreng, leaves were collected from local cultivar grown in Jayankondam (Ariyalur District, Tamil Nadu, India). The leafy portions were manually separated, and shade dried under room temperature condition. The moisture content of dried samples was estimated by toluene distillation method (ASTA, 1985) and further used for the extraction of flavonoids.

Sample extractions

Dried leaves (100 g) were coarse ground in a blender for about 10 s, and ground powder was weighed 1 g in a 50 ml polypropylene tube and extracted with 10 ml methanol (Romero et al., 2010). Chlorophyll was removed with ice cold acetone (1:20) and mixed thoroughly, and then chlorophyll layer was removed. The mixture was sonicated for 1 h at room temperature. After centrifugation, the supernatant was filtered through a 0.45 µm pore size cellulose membrane filter and the precipitate was re-extracted twice more following the same steps. The three filtrate supernatants were combined, from which 3ml was evaporated to dryness in a vacuum and the residue was re-dissolved in 500 µl of methanol, placed in 2-ml amber glass HPLC vials and analyzed by RP-HPLC coupled with DAD.

Reagents

HPLC-grade chemicals and reference compounds for authenticated standards (e.g. (±) rutin, quercetin, myricetin, kaempferol) were obtained from Fluka (Sigma-Aldrich; Bangalore, India), and were used to construct linear standard curves by injecting range of 20 to 200 ng. Flavonoids standards (Figure 1) were isolated and purified in lab with individual purity not less than 98% (HPLC assay, UV detection). Acids and bases were obtained by ACS quality. Filtered water was obtained from a Milli-Q Ultrapure water purification system (Millipore Pvt. Ltd, Mumbai, India). All the reference stock solutions were stored at -80°C. Diluted working solutions were freshly prepared for each HPLC analysis.

HPLC analysis for flavonoids

The HPLC system was equipped with an Agilent 1100 serial

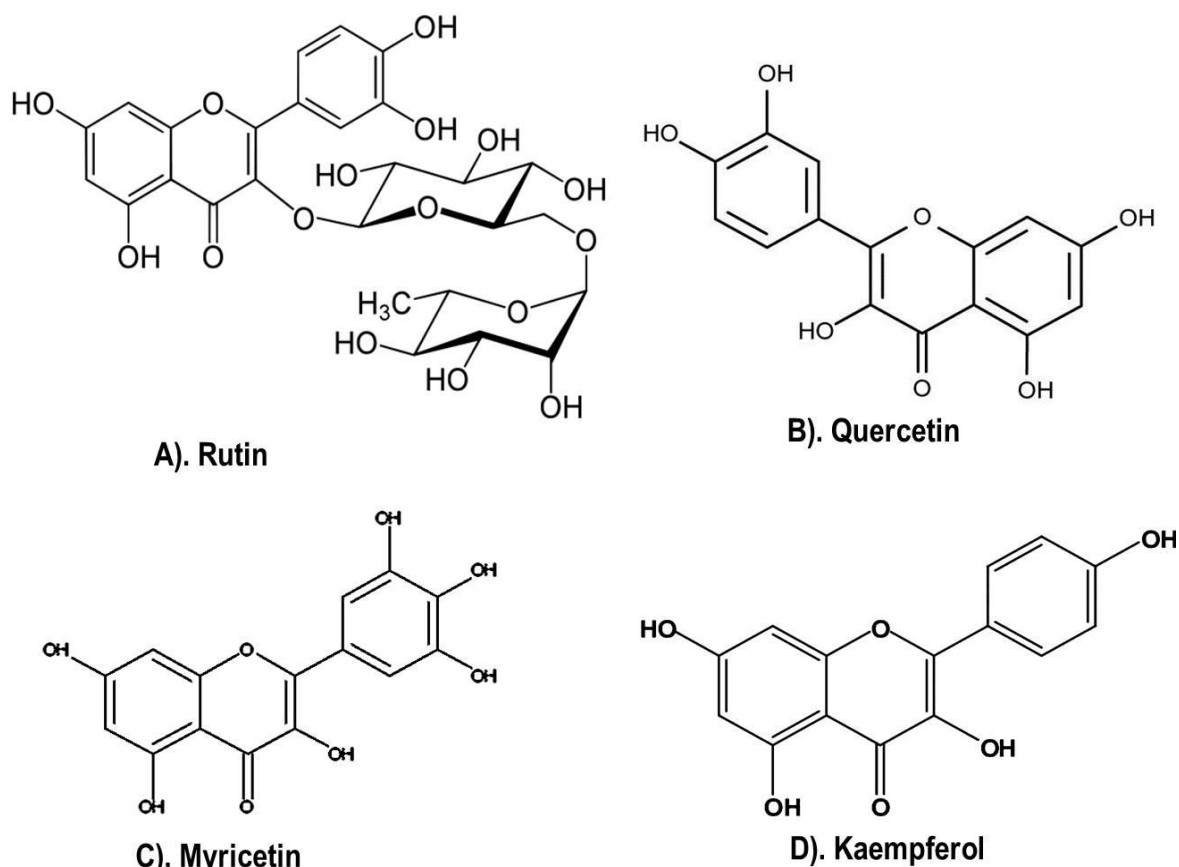


Figure 1. Chemical structure of Rutin, Quercetin, Myricetin, and Kaempferol.

system, consisting of a quaternary pump, online degasser, auto-sampler, column heater and variable wavelength detector. Separation was achieved on a reversed phase column (ZORBAX, Eclipse plus-C₁₈, 5 μ m, 4.6 \times 150 mm, Agilent, USA) preceded by a C₁₈ guard column at 40°C with a diode array detector (DAD) set at 190 to 600 nm and methanol:acetonitrile:water:acetic acid (40:20:39:1, v/v/v/v isocratically) employed as the eluent. The elution was kept constant at 0.8 ml/min and the peaks were simultaneously identified using UV absorbance at 350 nm for kaempferol and 254 nm for rutin, myricetin and quercetin. The injection volume was 10 μ l for each technical repeat. All the injections were performed in triplicate. The chromatographic peaks of the analytes were confirmed by comparing their retention time and UV spectra with those of the pure standards. Quantification was carried out by the integration of the peak using the external standard method.

RESULTS AND DISCUSSION

Range of linearity

The retention time of all the four individual analytes were presented in Table 1. The linearity was investigated for the authenticated four flavonoids standard by plotting peak area against the injected amounts. In the range of

20 to 200 ng, all the four standards were injected and good correlation of linearity has been achieved. The regression equation and correlation coefficient determined from the reference were presented in Table 2.

Reproducibility and accuracy

The study of reproducibility was performed on three consecutive days ($n = 9$) indicating a relative standard deviation of 3.50% for rutin, 3.25% for quercetin, 3.00% for myricetin and 3.36% for kaempferol. The accuracy of the analytical method was determined by assaying at least triplicate applications of each sample. The recovery test was used to evaluate the accuracy of the method. The average recovery ($n=5$) of quercetin and myricetin were both 99 and 98.2% for rutin and 99.2% for kaempferol. Relative standard deviation (RSD) was 2.77, 0.71, 1.58 and 2.17% for in rutin, quercetin, myricetin and kaempferol, respectively (Table 2). Analytes peaks were simultaneously identified using UV/Vis diode array detection at 350 nm for kaempferol and 254 nm for rutin, myricetin and quercetin (Figure 2). Typical chromatograms showed comparison between the kaempferol 40

Table 1. Identification of flavonoids by RP-HPLC analysis.

S/N	Compounds	Molar mass (g/mol)	Formula	RT ^a (min)	Lambda Max ^b
1	Rutin	610.52	C ₂₇ H ₃₀ O ₁₆	0.89	254
2	Quercetin	302.24	C ₁₅ H ₁₀ O ₇	1.29	254
3	Myricetin	318.23	C ₁₅ H ₁₀ O ₈	1.66	254
4	Kaempferol	286.23	C ₁₅ H ₁₀ O ₆	2.23	350

^aRT: Retention time; ^bLambda max, absorbance spectrum wavelength (nanometer).

Table 2. Standard calibration curve and average recovery percentage.

S/N	Compound	Standard purity (%)	Regression equation	Correlation (R ²)	Average recovery (%) ^a	RSD (%) ^b
1	Rutin	>98.0	4.4582 x -0.7012	0.9999	98.2	2.77
2	Quercetin	>98.0	5.6667 x -1.3192	0.9995	99.0	0.71
3	Myricetin	>97.0	5.2132 x -1.0153	0.9998	99.0	1.58
4	Kaempferol	>98.0	5.4721 x +0.2124	0.9999	99.2	2.17

^aAverage recovery: Observations (n=9); RSD (%): relative standard deviation (%).

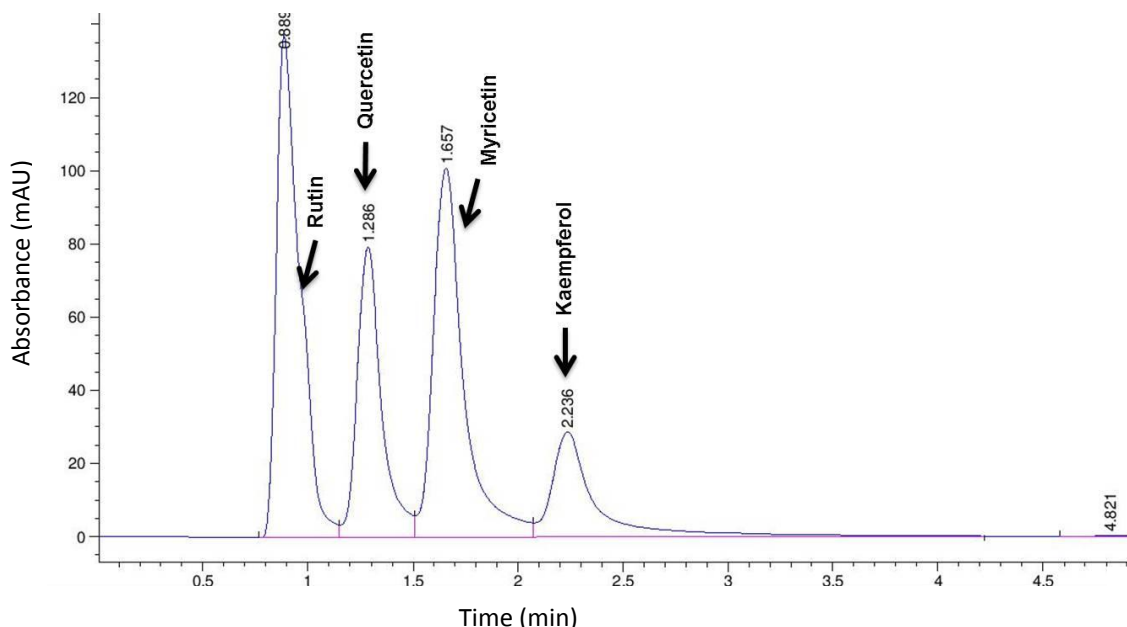


Figure 2. Chromatogram of external standards (catechin, quercetin, myricetin and kaempferol) concentration in 40 ng and absorbance at 254 nm.

ng concentration detected at 350 nm with highly significant greater peak area than 254 nm (Figures 2 and 3).

RP-HPLC analysis and quantitative determination of flavonoids

Three biologically replicated samples (nine technical

repeats) were extracted following the earlier mentioned procedure, and all injections were performed in triplicate analyzed in the Agilent HPLC system. The concentration of flavonoid analytes was determined by the regression equation. Total flavonoid concentrations were calculated by the sum of four individual flavonoids along with unknown flavonoids. The unknown flavonoids presented in the same chromatogram profile were calculated by using regression equation of rutin. HPLC assay was

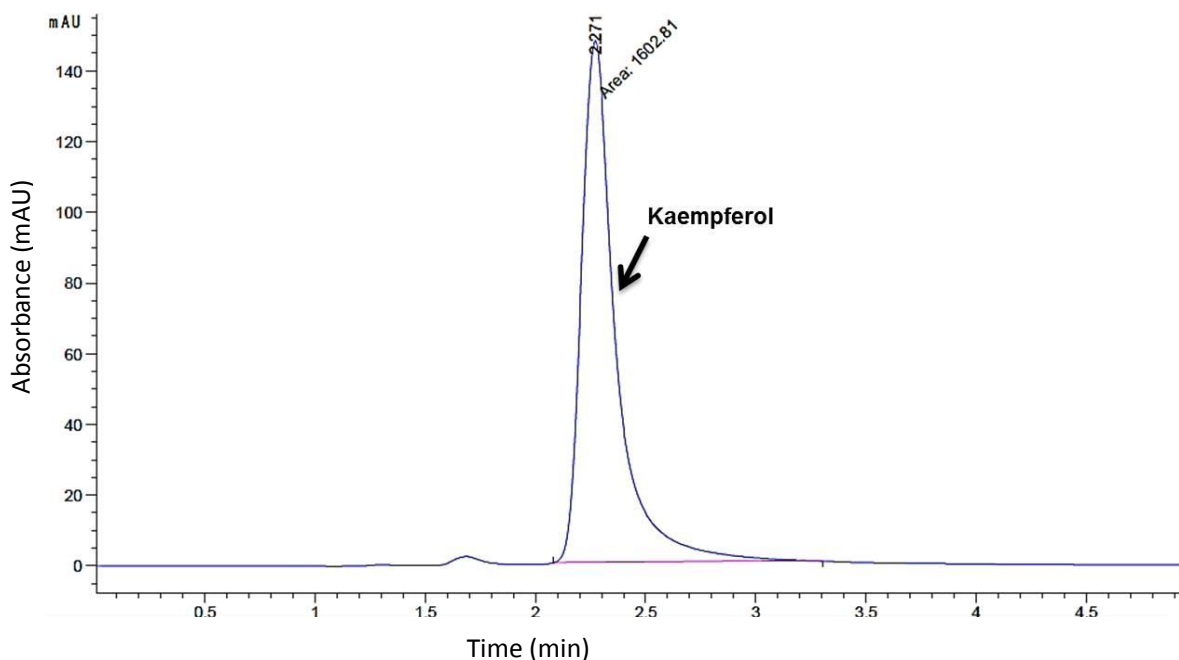


Figure 3. Typical chromatogram of external standard kaempferol (40ng) absorbance at 350 nm.

reproducible, sensitive and validated. Hence, it was successfully applied for the determination of rutin, quercetin, myricetin, and kaempferol in *M. koenigii* leaf samples. The results showed that three biologically replicated samples had similar retention time in HPLC chromatogram for all four individual analytes. Of the four analytes, mean value of rutin (924.25 mg/kg or 92.42 mg/100 g) and quercetin (85.88 mg/kg or 8.6 mg/100 g) accumulated greater in methanol extracts of *M. koenigii* leaves (Table 3 and Figure 4). Earlier study by Vijayand and Wesely (2011) reported *M. koenigii* leaves to contain quercetin and rutin and it confirmed our results.

In recent studies, it was reported that quercetin could inhibit LDL oxidation. In addition, the intake of quercetin greater than 4 mg/day has been found to have 21% reduction in cardiovascular disease mortality (Graf et al., 2005). Nevertheless, quercetin can also reduce inflammation by scavenging free radicals (Boots et al., 2008). Based on this report, our present study results to intake of 50 g/day, curry leaf was expected to produce approximately more than 4.0 mg of quercetin, and it will be helpful in reducing cardiovascular disease in human being. Flavonoid components act as the most powerful flavonoids for protecting the body against reactive oxidative species (ROS) damage (De Groot, 1994). Our study results showed that methanolic extract of curry leaf leaves have the huge amount of rutin. Hence, intake of antioxidants rich curry leaf will be more useful in protecting the human body against the oxidative stress damage by scavenging free radicals.

In this study, the RP-HPLC analysis also showed good resolution of peak separation of myricetin and kaempferol from *M. koenigii* leaves. The average value of methanolic extracts of curry leaf of myricetin and kaempferol are shown in Table 3. In addition, total flavonoids concentration was 1415.50 mg/kg (Figure 4). Kaempferol and myricetin are acting as antioxidants exhibited beneficial effects such as anti-inflammatory, antiallergic, antiviral as well as anticancer activity (Hillwell, 1994; Fraga et al., 1987; Ningappa et al., 2008). Dasgupta et al. (2003) observed that *M. Koenigii* leaves had significant reduction in tumor cells from Swiss albino mice and the blood-glucose levels of diabetic rats was treated with methanol extracts of curry leaf which was observed to have significant reduction as compared to diabetic control groups (Math and Balasubramaniam, 2005). Therefore, our study results observed that *M. koenigii* leaves have the natural abundance of rutin and flavonols, and it may exhibit several health benefits.

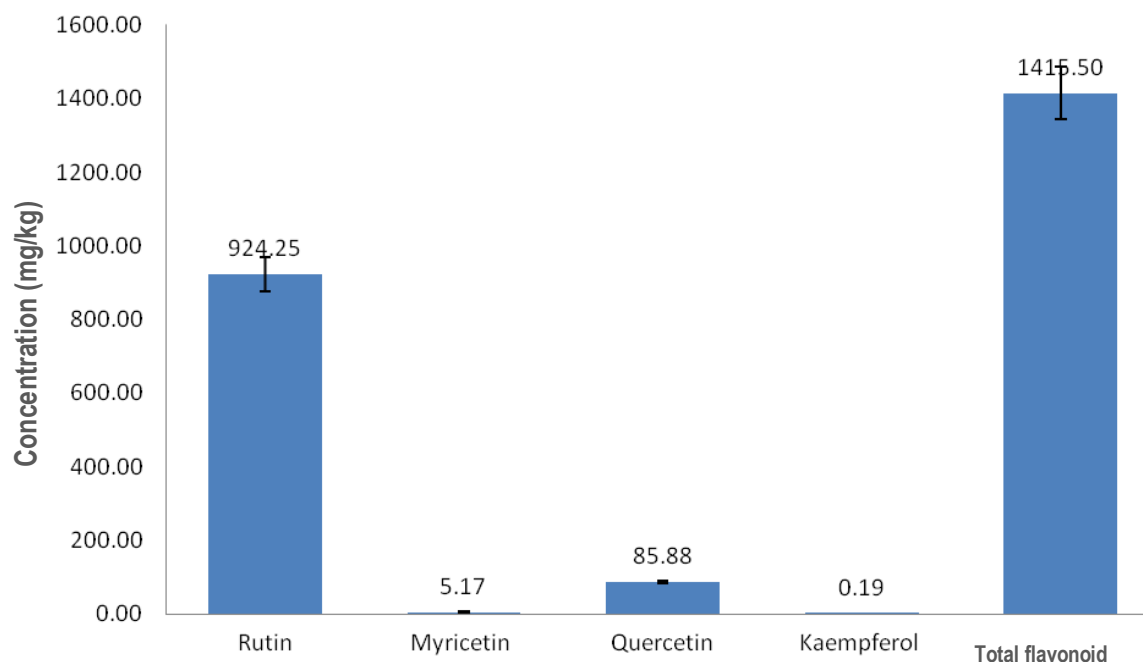
Conclusion

Methanol extracts of curry leaf contains rutin, quercetin, myricetin and kaempferol identified by RP-HPLC-DAD analysis. The individual flavonoid concentration was determined by the authenticated reference analytes regression equation, and assay was reproducible, sensitive and validated. The analysis revealed that rutin and quercetin are subjected to the presence predominantly,

Table 3. Quantification of flavonoid concentration in *Murraya koenigii* leaves.

S/N	Flavonoid	mg/kg ^a	SD ^b	SE ^c
1	Rutin	924.25	3.77	1.26
2	Myricetin	5.17	0.15	0.05
3	Quercetin	85.88	4.84	1.61
4	Kaempferol	0.19	0.04	0.01
5	Total flavonoids	1415.50	7.01	2.34

^amg/kg: Milligram per kilogram; ^bSD: standard deviation (n=9); ^cSE, Standard error.

**Figure 4.** Flavonoid concentration in curry leaf (*Murraya koenigii*) leaves with 5% error bar.

whereas myricetin and kaempferol concentrations were accumulated with significant amount in curry leaf leaves. This study suggests that accumulation of greater amount of bioactive components like rutin and quercetin in *M. koenigii* leaves will provide more useful information for further pharmacological investigations.

ABBREVIATIONS

BHT, Butylated hydroxytoluene; **BHA**, butylated hydroxyanisole; **DAD**, diode array detector; **RP-HPLC**, reverse phase high performance liquid chromatography.

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