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Antioxidants properties of *Murraya koenigii*: a comparative study of three different extraction methods

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Abstract

Nutraceutical and pharmaceutical industries have been increasingly engaged in finding natural alternatives compounds as potential antioxidants. The use of phytochemicals is introduced as a good source of natural antioxidants. *Murayya koenigii* leaves, commonly used in cooking and traditional medicines have been examined for their remarkable antioxidant potential, yet still, it remains an understudied herb. Therefore, this study aimed to determine the antioxidant properties and flavonoids profile in *M. koenigii* leaves extracted using; solvent assisted extraction (SAE), microwave assisted extraction (MAE) and ultrasonic assisted extraction (UAE). The antioxidant properties of *M. koenigii* were analysed qualitatively and quantitatively using high performance liquid chromatography (HPLC). *M. koenigii* leaves extracted using the UAE method have responded strongly towards a 2, 2-diphenyl -2-picryl-hydrazyl DPPH assay with the highest inhibition (%) of 78.00±1.00. Using the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) method assays, the *M. koenigii* leaves with the lowest absorbance were assigned as a sample with the highest antioxidant activity. The *M. koenigii* leaves extracted using UAE had the lowest absorbance with 0.01±0.00. In the TPC assay, the MAE method showed the highest total phenolic content (120.60±14.81 mg GAE/g sample). The TFC assay demonstrated that MAE methods have the highest total phenolic content (93.38±4.33 mg QE/g sample. The *M. koenigii* leaves extracted by MAE showed the highest gallic acid, catechin, epigallocatechin gallate, rutin and kaempferol concentration (mg/L). *M. koenigii* leaves subjected to SAE extraction has the highest concentration of p-coumaric acid, myricetin and quercetin (mg/L). This study found that *M. koenigii* leaves extracted using UAE exhibited better antioxidant activities than that of MAE and SAE. These useful findings have managed to narrow the knowledge gap regarding the effects of different extraction methods on the antioxidant property of *M. koenigii*.

1. Introduction

In recent times, medicinal plants are on a rave due to their variant physicochemical properties. These natural products have been shown to have antioxidant properties. They are capable of scavenging free superoxide radicals, thus providing anti-ageing benefits as well as reducing the risk of cancer (Ghasemzadeh *et al*., 2014). A significant role of dietary phytochemicals human health is mainly to minimize oxidative damage to living cells by deactivating reactive oxygen species (ROS), the by-products produced during normal cell aerobic respiration (Dharmaraja, 2017). Many studies have been carried out on many local herbs in Malaysia; however, there are still some other herbs such as *M. koenigii* leaves that remain unexplored in depth and more explorations are needed (Azizah *et al*., 2014).

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The leaves of *Murraya koenigii* have been widely used in Indian cookery for centuries and have a versatile role to play in traditional medicine (Jain *et al.,* 2012). The *M. koenigii* leaves are notable for their antitumor, antioxidant, anti-inflammatory, anti-hyperglycemic, and hypoglycemic properties (Dineshkumar *et al*., 2010). The leaves have a slightly pungent, bitter and weakly acidic taste. They also retain their flavour and other qualities even after drying (Sinha *et al*., 2012). *M. koenigii* leaves are generously credited with tonic and stomachic properties. The bark and roots are used as a stimulant and topically to cure eruptions and bites of poisonous animals (Singh *et al*., 2014). The fresh leaves, dried leaf powder, and essential oils are widely used for flavouring soups, curries, fish and meat dishes, egg-based dishes, traditional curry powder blends, seasoning and other ready to use food preparations (Jain *et al.,* 2012). Traditionally, *M. koenigii* leaves are boiled with coconut oil until they are reduced to a blanked residue which is then used as an excellent hair tonic for retaining natural hair tone and stimulating hair growth.

In the nutraceutical industry, the extraction process is an important step for the isolation of phytochemicals from herbs and spices (Bak *et al*., 2012). However, there are some disadvantages with the use of certain types of extraction method such as environmental pollution, lower yields, loss of reactivity, and others. Conventional solvent extraction attracts a higher cost, requires a longer time and is inefficient (Luque-Garcia and Luque de Castro, 2003), therefore alternative extraction needs to be explored to reduce extraction time, reduce solvent consumption, increase extraction yield and improve extract quality. This problem could be hindered by the use of an efficient extraction technique to preserve the beneficial properties of the extract. A comparison with other extraction techniques to obtain beneficial compounds proved to be crucial and important (Medina-Torres *et al*., 2017). Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are recommended for the extraction of analytes from different matrices. The advantages of UAE and MAE are low equipment cost, low energy requirements, reduced solvent quantity and/or time consumption (Jacotet-Navarro *et al*., 2016). Therefore, the purpose of this study was to determine the antioxidant properties and flavonoid content in *M. koenigii* leaves extracted using different extraction procedures namely, solvent assisted extraction SAE), microwave assisted extraction (MAE) and ultrasonic assisted extraction (UAE).

2. Materials and methods

All samples were dried using the oven drying method (AOAC, 2007) prior to all extraction techniques. The samples were then ground and sieved (10-20 mm size) (Waring Commercial, Torrington. CT, U.S.A) and kept in a dark bottle, stored at 5^{o}C until further analysis.

2.1 Solvent assisted extraction

Solvent assisted extraction was conducted using 60% ethanol using a modified method suggested by Zainol *et al*. (2003). Approximately 10 g of the finely ground curry leaf powder was added with 100 mL of 60% ethanol and left to stand for 1 hr. The solvent was then removed leaving the residue for the second extraction. The solvent was filtered using Whatman No. 4 filter paper using a vacuum pump. Post filtration the filtrate was then concentrated using a rotary evaporator (Buchi, Switzerland).

2.2 Microwave assisted extraction

Microwave assisted extraction was conducted using a modified method suggested by Dahmoune *et al*. (2015). The solution was prepared according to a 28:1 solvent to solid ratio, where 10.7 g of dried curry leaf powder was mixed with 300 mL of 60% ethanol. The solution was then irradiated for 3 min using a microwave set to 300W (Samsung, Korea).

2.3 Ultrasonic assisted extraction

Ultrasonic assisted extraction was conducted using a modified method suggested by Zainol *et al*. (2018). A total of 38 g powdered *M. koenigii* leaves were mixed with 60% ethanol. The mixture was submerged in an ultrasonic cleaner bath (Buchi, Switzerland) and extracted for 30 min. The extracted samples were then centrifuged at 700 rpm at 4°C for 10 mins. Ethanol was then removed from the extract using a rotary evaporator and the resulting extract was placed in a bottle in the chiller for the next analysis.

2.4 Determination of antioxidant properties

2.4.1 2, 2 -diphenyl -2-picryl-hydrazyl (DPPH) method

The capacity of trapping of free radical DPPH was evaluated according to the method described by Zainol *et al*. (2018) with slight modification. A 6.1 × 10^{-5} M solution of DPPH was prepared in ethanol. Then, 75 µL of the diluted extract was added to 3 mL of the DPPH solution. The absorbance was taken at 515 nm using methanol with DPPH as the negative control whilst quercetin was the positive control after letting it stabilise for 1 hr. All operations or conducted in dark or dim light. The inhibition percentage (IP) of the DPPH by the extract was calculated according to the formula:

IP = [(A0 min - A60 min) / A0 min] x 100

A0 min is the absorbance of the blank at t = 0 min, and 60 mins is the absorbance of samples at 60 mins. The result was expressed as μmol Trolox equivalent (TE) per gram of sample on a dry basis, through a dose-response curve for Trolox (0-350 μM).

2.4.2 Ferric thiocyanate (FTC) method

The samples were analysed using methods suggested by Zainol *et al*. (2003). The adjusted 1mg/mL of sample was dissolved in 4 mL of absolute ethanol (99.5%), added with 4.1 mL of 2.52 % linolenic acid in absolute ethanol. Eight millilitres mL of 0.05M phosphate buffer (pH7) were mixed with 3.9 mL of distilled water and kept in a screw cap bottle and placed in a water bath shaker at 40°C. Approximately 0.1 mL of samples were added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate finally by 0.1 mL of 0.02M ferrous chloride added in 3.5% Hydrochloric acid into the reaction mixture. The absorbance of the resulting red -blood colour was measured after 3 mins at 500 nm every 24 hrs until the day the absorbance of the control reached the maximum value.

2.4.3 Thiobarbituric acid (TBA) method

The samples were analysed using methods suggested by Malik *et al*. (2017). An aliquot (1 mL) of sample solution obtained by the FTC method was added to 2 mL of 20% thrichloroacetric acid and 2 mL of 0.67 % 2-thiobarbituric acid. The mixture was placed in boiling water at 100°C for 10 mins. Next, the mixture was cooled and then centrifuged at 300 rpm for 20 mins. The absorbance of the supernatant was measured at 552 nm.

2.4.4 Determination of total phenolic content (TPC)

The total phenolic compounds were determined using Folin-Ciocalteu reagent according to the colourimetric method described by Ng *et al*. (2020). An aliquot (1 mL) of every sample was diluted into 50 mL of stock solution. Approximately 1 mL from the stock solution was added to 17.9 mL of distilled water in 0.5 mL of Folin-Ciocalteu reagent and left to stand for 1 min. Then, 1.5 mL of 20% sodium carbonate was added to the mixture. The sample prepared was then left at room temperature for 2 hrs in the dark. The absorbance value was taken at 765 nm, resulting in mg GAE per gram of sample extract (mg GAE/g) expressed as gallic acid equivalent.

2.4.5 Determination of total flavonoid content (TFC)

Approximately 50 mg of sample was mixed with 1.5 mL methanol, 0.1 mL 10% aluminium chloride, 0.1 mL 1M potassium acetate and 2.8 mL distilled water. The mixture was then incubated at room temperature for 30 mins. The absorbance of the reaction mixture was taken at 415 nm. The result was expressed as mg of quercetin equivalent (QE) per gram of sample extract (MG QE/G) as mg quercetin equivalent (QE)/g of dried plant material (Chong *et al*., 2018).

2.4.6 Determination of individual flavonoid content using HPLC

All samples were refluxed in 6 M HCl at 90°C for 2 hrs with 60% (v/v) aqueous methanol extracted prior to the HPLC analysis (Mohd Zainol *et al*., 2009). HPLC analysis was performed using an Analytical High - Performance Liquid Chromatography (HPLC) (Shimadzu, Japan) with a 4 solvent delivery system quaternary pump (LPG 3400 SD) including a diode array detector (DAD 3000) with 5 cm flow cell, a manual sample injection valve equipped with a 20 μL loop and Chromeleon 6.8 system manager as the data processor. A total of 20 mL of sample were injected into the HPLC system for every analysis. One percent (V/V) aqueous acetic acid solution and acetonitrile were used as the gradient mobile phase. The flow rate was fixed at 0.7 mL/min, while the column oven was set at 28°C. The composition of the mobile phase was back to the initial condition (solvent B: solvent A: 10: 90) in 31 mins and allowed to run for another 5 mins before the injection of the next sample. HPLC chromatograms were detected using a photodiode array UV detector at 270 nm. Each compound was identified by its retention time and by spiking with standards under the same conditions (Seal, 2016).

2.5 Statistical analysis

The data obtained were subjected to one-way analysis of variance (ANOVA). The mean comparisons from triplicate analysis were carried out using Fisher's Least Significant Difference (LSD) test (Mamat *et al*., 2018). Statistical analysis was performed using SPSS software 2004.

3. Results and discussion

3.1 Extraction yield from solvent assisted extraction, microwave assisted extraction and ultrasonic assisted extraction

Table 1 shows the extraction yield (%) of three different extraction methods. The extraction yield of solvent assisted extraction (SAE) showed no significant difference compared to the extraction yield of ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE).

Table 1. Total phenolic content, total flavonoid content, ABTS inhibition and DPPH inhibition of the SCG extracted using different extraction methods of *M. koenigii* leaves

	Control	SAE	MAE	UAE	BHT	Vit C	α-Tocopherol
Extraction yield (%)	-	1.47±0.04^{b}	1.60±0.01^{ab}	1.70±0.01^{a}	-	-	-
DPPH (%)	5.20±0.62^{c}	63.67±3.22^{b}	75.33±1.53^{ab}	78.00± 1.00^{ab}	88.67±1.16^{a}	80.38±3.88^{ab}	79.32±3.88^{ab}
FTC (Abs)	0.6±0.02^{a}	0.2±0.01a^{b}	0.16±0.02^{b}	0.13±0.02^{b}	0.11±0.02^{b}	0.13±0.01^{b}	0.14±0.01^{b}
TBA (Abs)	0.95±0.02	0.34±0.02^{a}	0.27±0.03^{b}	0.25±0.01^{b}	0.16±0.01^{c}	0.21±0.01^{b}	0.23±0.01^{b}
TPC (mg GAE/g)	-	58.48±5.46^{b}	120.60 ±14.81^{a}	88.79±4.48^{b}	-	-	-
TFC (mg QAE/g)	-	27.97±7.12^{c}	85.23±2.35^{a}	66.45±5.92^{b}	-	-	-

Solvent-assisted = SAE, Microwave assisted = MAE, Ultrasonic assisted = UAE. Values represent the mean±standard deviation. Values with the same letter superscript are not significantly different (P<0.05) between samples within rows.

The increasing trend of the extraction yield can be expressed in such a manner where the extraction yield of UAE (1.70±0.10%) > MAE (1.60±0.01%) > SAE (1.47±0.04%). Tiwari (2015) stated that UAE is based on the concept of acoustic cavitation capable of damaging the cell wall of the plant matrix and thus favouring the release of bioactive compounds. This method can be used to extract a wide variety of phytochemicals such as phenolic compounds, indicating that it is the best technique for achieving higher yields. The exceptional capability of the acoustic cavitation to damage the cell wall in ultrasonic assisted extraction (UAE) encourages the release of more phytochemicals, thus increasing the extraction yield in this particular extraction method (Ledesma-Escobar *et al*., 2015).

3.2 2, 2 -diphenyl -2-picryl-hydrazyl (DPPH) method

Table 1 also shows that there was no significant difference between MAE and UAE extracts of *M. koenigii* leaves, α-tocopherol and vitamin C. The data show that *M. koenigii* leaves subjected to the UAE method reacted strongly with the DPPH assay (78.00±1.00%) followed by the MAE (75.33±1.53%) and the SAE (63.67±3.22%). This can further illustrate the radical scavenging properties of the curry leaf extracts obtained by different methods, where UAE had the highest radical scavenging properties followed by MAE and SAE, as they show higher inhibition (%) against oxidation. Similarly, strong radical scavenging properties of the UAE method have been demonstrated by the Bouaoudia-Madi *et al*. (2019) pericarp study of *Myrtus communis*, where the results show that the UAE extract showed higher DPPH Scavenging capability compared to the SAE and MAE extracts.

3.3 Ferric thiocyanate method (FTC)

The ferric thiocyanate (FTC) value of the UAE extract did not show a significant difference compared to butylated hydroxytoluene (BHT), Vitamin C and α-Tocopherol (Table 1). This could indicate that the UAE method extracts reacted strongly to the FTC assay in addition to the DPPH assay. As the lowest absorbance reading on the fourth day of incubation dictates the highest antioxidant quality. UAE methods have been shown to preserve more of the antioxidant quality of *M. koenigii* leaves followed by MAE and SAE methods. The antioxidant quality demonstrated by the samples mimics a number of studies previously conducted. According to Jun *et al*. (2011), the crude extracts of green tea from the UAE method showed a high radical scavenging capability followed by MAE and SAE. The FTC assay also agrees with previous antioxidant quality patterns demonstrated by the UAE DPPH assay antioxidant quality is the highest compared to MAE and SAE.

3.4 Thiobarbituric Acid (TBA) method

The MAE extracts showed the lowest absorbance, indicating that they had the highest antioxidant quality compared to the SAE and UAE methods in the TBA assay (Table 1). Dahmoune *et al*. (2015) reported that citrus lemon extracts produced using MAE had higher lipid peroxidation compared to SAE and UAE extracts. This explains why MAE peroxidation is lower compared to SAE and UAE methods. Ince *et al*. (2012) also explained that MAE performed better due to better inhibition of lipid peroxidation by better antioxidant activity against hydroperoxide and free radical formation.

3.6 Total phenolic content (TPC) method.

M. koenigii leaves extracted using the MAE method showed the highest total phenolic content (TPC) (120.60±14.81 mg GAE/g sample), followed by UAE (88.79±4.48 mg GAE/g sample) and SAE (58.48±5.46 mg GAE/g sample). Compared to previous assays, it can be observed that the antioxidant activity of the UAE method in TPC was not as favourable as in previous analyses such as DPPH and FTC assays. This could be due to the high frequency in the UAE that could easily degrade the TPC. Degradation of flavonoids and polyphenol compounds is possible at high frequencies in the UAE (Jahromi, 2019). Routray and Orsat (2012) also reported that for total phenolic extraction, microwave

applications were observed to have a much higher overall phenolic output than sonication-based extraction.

3.7 Total flavonoid content (TFC) method.

Table 1 shows that there is a significant difference between the total flavonoid content (TFC) of *M. koenigii* leaves of three different MAE, UAE and SAE extracts. A similar trend was observed in the TPC analysis. However, the total flavonoid content of *M. koenigii* leaves showed a better significant difference compared to the TPC of *M. koenigii* leaves. The data suggest that *M. koenigii* leaves respond better to oxidation induced by aluminium flavonoid complexes. The total flavonoid content of *M. koenigii* leaves extracted by MAE was also found to be the highest in comparison to UAE and SAE. Analogous results have also been established by Nouha *et al*. (2017) on Maltese orange peel, where the method with the highest total phenol and flavonoid content is microwave-assisted extraction followed by ultrasound-assisted extraction, conventional solvent extraction.

3.8 Determination of individual flavonoids using HPLC analysis.

Figure 1 shows the HPLC chromatogram which verifies the presence of the designated standard flavonoid used in this study, identified by its retention time, while Figure 2 shows the HPLC chromatogram confirming the presence of individual flavonoids in the sample undergoing different extraction treatments, in line with the flavonoid standards shown in Figure 1. The results show that rutin, myricetin, kaempferol, gallic acid, catechin, epigallocatechin gallate, quercetin and p-coumaric acid were present after all types of extractions. These figures indicate the presence of the compounds tested confirming that the HPLC technique used was suitable for the detection, identification and measurement of the availability of flavonoids (Moniruzzaman *et al*., 2014).

Table 2 shows the flavonoid content in all three samples of *M. koenigii* leaves from three different extraction methods (SAE, MAE and UAE) determined using an HPLC analysis. All samples analysed showed the presence of catechin, epigallocatechin gallate, quercetin, gallic acid, myricetin, kaempferol, rutin and p-coumaric acid (Figure 2). The previous study on *M. koenigii* leaves by Sepahpour *et al*. (2018) indicated that the 80 %t ethanol extraction yielded 0.9, 5.4, 2.4 and 1.4 mg/g freeze- crude extract of rutin, quercetin-3-glucoside myricetin and quercetin. It is interesting to note that the different extraction techniques used in the study affected the individual flavonoids differently. Results of the study also found that *M. koenigii* leaves extracted by MAE had the highest concentration of total individual flavonoids, while SAE showed the lowest concentration of total individual flavonoids between samples. Catechin was found to be the concentration compound found in all of the extracts analysed. In contrast, Hertog *et al*. (1992) reported that quercetin is the major flavonol found in vegetables such as broccoli, kale, French beans, celery, onions and cranberries. In addition, rutin was found to be the least abundant flavonoid in all the samples tested.

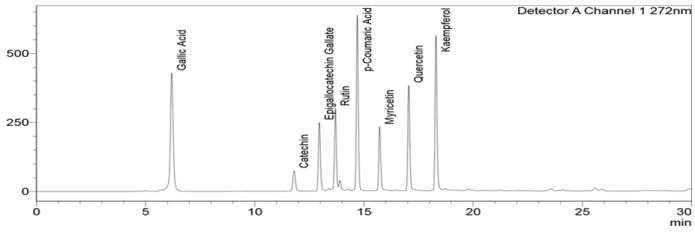

Figure 1. The mixed standards of the HPLC chromatogram

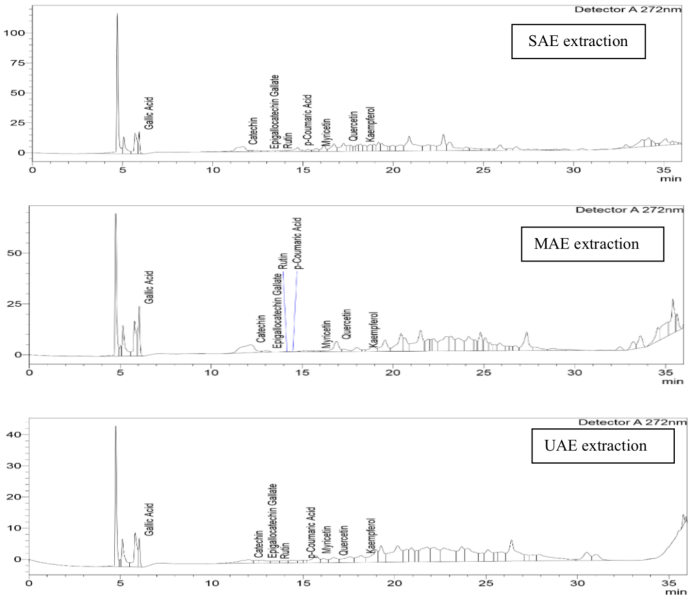

Figure 2. HPLC chromatogram of the *M. koenigii* leaves extracted using SAE, MAE and UAE

Table 2. The concentration of individual flavonoid in *M. koenigii* leaves extracted using different extraction techniques (SAE, MAE and UAE)

	The concentration of the individual flavonoids in every sample (mg/L)							
	Gallic acid	Catechin	Epigallocatechin	Rutin	p-coumaric	Myricetin	Quercetin	Kaempferol
SAE	0.16±0.01^{b}	1.82±0.05^{b}	0.10±0.01^{b}	0.05±0.00^{a}	0.19±0.02^{a}	0.31±0.01^{b}	0.50±0.02^{a}	0.15±0.01^{a}
MAE	0.27±0.01^{a}	2.29±0.02^{a}	0.85±0.06^{a}	0.06±0.01^{a}	0.01±0.00^{c}	0.46±0.03^{a}	0.30±0.02^{b}	0.17±0.01^{a}
UAE	0.18±0.01^{b}	0.83±0.05^{c}	0.23±0.01^{b}	0.07±0.01^{a}	0.07±0.00^{b}	0.13±0.01^{c}	0.22±0.02^{b}	0.11±0.00^{a}

Solvent-assisted = SAE, Microwave assisted = MAE, Ultrasonic assisted = UAE. Values represent the mean±standard deviation. Values with the same letter superscript are not significantly different (P<0.05) between samples within rows.

The different results obtained from the different extraction procedures observed between flavonoids could arise from the different properties embedded within each type of flavonoid (Mohd Zainol *et al*., 2009).

4. Conclusion

M. koenigii leaves extracted using UAE exhibited better antioxidant activity than that of MAE and SAE. The analyses of TPC and TFC showed that the MAE extract *M. koenigii* leaves showed the best results compared to the UAE and the SAE. *M. koenigii* leaves extracted by MAE exhibited the highest total number of individual flavonoids compared to *M. koenigii* leaves extracted using UAE and SAE had the highest concentration of p-coumaric acid, myricetin and quercetin concentration (mg/L). Catechin was the highest flavonoid detected in all the different extraction methods used in the study. The main strength of this study is that it narrows the current discrepancy between the impacts of different extraction methods on the antioxidant properties of *M. koenigii*.

Conflict of interest

The authors declare that there is no conflict of interest in the conduct of this study.

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