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Antioxidative effect of *Melastoma Malabathticum* L Extract and Determination of its Bioactive Flavonoids from Various Location in Malaysia by RP-HPLC with Diode Array Detection

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ABSTRACT

A rapid and specific RP-HPLC method with DAD at 30°C was developed and validated for the determination of quercetin (QN) and kaempferol (KMP) as marker compounds in the extract of *Melastoma malabathrichum* leaves. Separation was performed using Hypersil GOLD C8 column with acetonitrile - 0.1% phosphoric acid (40:60v/v) as a mobile phase with DAD detection at 370 nm. The developed method showed satisfactory reproducibility and recovery and the method was successfully applied in the determination of QN and KMP as marker compounds in MM leaves collected from different parts of Malaysia and was found to be simple, rapid and efficient. The antioxidant activity of MM of 3 different solvent extracts (methanol, ethyl acetate, chloroform) were evaluated using β-carotene bleaching method. A variation in antioxidant activities, ranging from 44.41% to 83.28% was observed and the antioxidative potency of the 3 different solvent extracts was comparable to that of butylated hydroxytoluene (BHT). The results showed that MM extracted by different solvents exhibited varying degree of antioxidant properties.

INTRODUCTION

The antioxidative properties of medicinal plants create interest of many researchers. The oxidative modification of protein, lipids, DNA and small cellular molecules by reactive oxygen species plays significant role in a whole wide range of common diseases and age related degenerative conditions such as neurodegenerative disease, wrinkled skin, DNA damage, cardiovascular disease, inflammatory conditions as well as carcinogenesis (Takashi et al., 2007). Recently, there has been considerable interest in finding naturally occurring antioxidants to replace synthetic antioxidants in foods.

The addition of synthetic antioxidants in food and beverage may impose health risks resulting in strict regulations over their use in foods (Hettiarachchy et al., 1996). Several studies have been carried out to analyze the antioxidant potential of a variety of herbs and the most active principle among the phytochemicals is the phenolic fraction (Akowuah et al., 2004). One such herb which contains polyphenols (mainly flavonoids and tannins) is *Melastoma malabathricum* (Susanti et al., 2007). *Melastoma malabathricum* (MM) is a plant from the family Melastomaceae commonly found in previously cleared land, waste places, and roadside throughout the Southeast Asian countries, including Malaysia (van Valkenberg et al., 2001). MM consists of three varieties, having flowers with dark purple-magenta petals, light pink-magenta petals and the rare variety of white petals (Susanti et al., 2007). Generally, various parts of the plant are used in Malay, Indian, and Indonesian folk medicines to treat various types of ailments and diseases including diarrhea, dysentery, leucorrhoea, haemorrhoids, cuts and wounds, infection during confinement and also used to prevent scarring of smallpox and to treat piles (Begum and Nath, 2000; Ong and Norzalina, 1999). Despite being a traditional medicinal herb that is widely used, particularly, in Malay culture, there is not much scientific study carried out on *M. malabathricum* (Mohd. Joffry et al., 2011).

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This could limit the use of this plant as a potential medicinal remedy. There are several reports on the isolation and identification of phytochemical constituents from this plant. Among the pythochemicals reported are ellagic acid, cyanidin-3-glucoside, cyanidin-3,5-diglucoside, malvidin-3,5-diglucoside, *β*-sitosterol, ursolic acid, 2-hydroxyursolic acid, gallic acid, kaempferol, kaempferol-3-*O*-*α*-L-rhamnopyranoside, malabathrin B, malabathrin C, malabathrin D, strictinin, (-)-epicatechin, *α*-amyrin, quercetin, quercitrin and rutin (Lowry, 1968; Lowry, 1976; Manzoor et al., 1981; Dinda and Saha, 1986a; Dinda and Saha, 1986b; Dass and Kotoky, 1988; Dinda and Saha, 1988; Yoshida et al., 1992a; Yoshida et al., 1992b; Nuresti et al., 2003; Wong et al., 2004; Nazlina et al., 2008; Susanti et al., 2008). However, there is no report on the separation of bioactive flavonoids from this plant using HPLC. To date, a comparative study on the content of the bioactive constituents of MM covering the regions of Malaysia has not been done. As such the present study has been undertaken to develop a HPLC method to separate, determine and to compare the content of quercetin and kaempferol which are known for their antioxidant activities as biomarker compounds in the leaves of MM from various locations in Malaysia. The anti-oxidative property of the different solvent extracts of MM was evaluated using β-carotene bleaching method.

EXPERIMENTAL

Plant material

Leaves of MM with light pink-magenta flower were collected from Semeling, Kedah, Malaysia. Voucher plant specimen (Voucher No.11088) were identified and deposited at the Herbarium of School of Biological Sciences, Universiti Sains Malaysia (USM). MM leaves from twelve different locations in the northern part of Malaysia were also collected and identified for the quantification studies.

Chemicals

Kaempferol, quercetin, linoleic acid and β-carotene was obtained from Sigma (St. Louis, MO, USA). All chemicals and solvents used in the assay procedure were of analytical/chromatography grade. Methanol, acetonitrile, glacial acetic acid, ethyl acetate, chloroform and phosphoric acid were purchased from Merck (Darmstadt, Germany).

Sample preparation for HPLC analysis

The fresh leaves of MM collected from various locations were cleaned and dried at 60ºC. The extraction was carried out using 2 g of powdered leaves with 30 mL of 85% methanol under 80 kHz, 45ºC in an ultrasonic extraction device for 30 min, repeated for twice. The extract was collected and filtered and the filtrate was dried at 50ºC under reduced pressure in a rotary evaporator. The dried extract was dissolved in the mobile phase. After filtering through a filter paper and a 0.45µm membrane filter (Millipore), the extract was injected directly into HPLC system.

Sample preparation for antioxidant analysis

The MM leaves collected from Semeling, Kedah were washed, air dried for 3 to 4 days and grinded into powder. Three hundred grams of weighed powder was extracted in a soxhlet apparatus with chloroform, followed by ethyl acetate and finally with methanol. The extracts were then filtered using Whatman No.1 filter paper and the filtered were then evaporated to dryness under pressure using rotary evaporator. The percentage yield of the extracts for chloroform, ethyl acetate and methanol were 95.4, 108.7 and 91.2 mg/g dry material respectively. The extract was stored in labelled sterile screw capped bottle and kept in -20°C until analysis.

Preparation of standard solutions

Standard stock solutions of quercetin and kaempferol were prepared in methanol, each at a concentration of 0.1 mg mL^{-1} respectively. All the standard solutions were kept in -20 ºC and filtered through 0.45 µm membrane filter (Millipore) prior to injection into HPLC system.

Chromatographic conditions

HPLC analysis was performed using Shimadzu Corporation HPLC equipped with reservoir tray, Prominence Degasser (DGU-20A5), Prominence Liquid Chromatography (LC-20AD), Prominence Communication Bus Mobile (CBM-20A), Prominence Diode-Array Detector (SPD-M20A), Prominence Auto Sampler (SIL-20ACHT), Column Oven (CTO-10 AS VP) was used. Chromatographic analysis was carried out by Hypersil GOLD C8, 250×4.6 (mm), reversed phase column packed with 5 µm diameter particles with the mobile phase consists of acetonitrile - 0.1% phosphoric acid (40:60, v/v). The mobile phase was filtered through a 0.45 µm membrane filter (Millipore), then de-aerated ultrasonically prior to use. Quercetin (QN) and kaempferol (KMP) were used as markers. The temperature was maintained at 30°C, with injection volume of 50 µL and flow rate of 1 mL/min. The effluent was monitored at 370 nm using diode array detector. Standard calibration curves were established by plotting of the areas of peaks against different concentrations of the reference compounds (varying from 0.1-100 ug/mL for both QN and KMP). The chromatographic peaks of the leaf extracts from different locations were confirmed by comparing their retention time and UV spectra with the reference standard. The system suitability of the method was evaluated by the intra- and inter-day precision and accuracy of replicates. The accuracy was evaluated through recovery studies by adding known amounts of the standard solution to the extract. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three concentrations of the standards (Ravichandran et al., 2010).

Sample solution stability

The stability of the QN and KMP in standard solution (three different concentration) and extract sample during analysis was determined by repeated analysis of samples during the course

of experimentation on the same day and everyday for one week and thereafter for week 2, 3 and 4 under laboratory bench conditions (25 ± 1°C) and under refrigeration (4 ± 0.5°C).

Determination of antioxidant activity using β-carotene bleaching method

The antioxidant activities of the samples were carried out by using linoleic acid system reported by Obame et al., 2008, with slight modification. A stock solution of β-carotene-linoleic acid mixture was prepared as stated: 0.5 mg of β-carotene was dissolved in 1 mL of chloroform, 25 µL of linoleic acid and 0.2 mL of Tween 20. The stock solution was heated up in a water bath by controlling the temperature at 30 - 40°C for 10 minutes to remove the chloroform. Hundred milliliters of oxygen-saturated distilled water was added into the stock solutions prepared and it was shaken with orbital shaker at 285 rpm. The stock solution was named as reagent A. The different solvent extracts of MM were diluted to 200 µg/mL equivalent to 0.004 g/20 mL. Five milliliters of reagent A was added into the test tubes using micropipette (with 5 mL scale) with 700 µL of the extracts with micropipette (with 1 mL scale). The samples to be tested were incubated using water bath at 50°C throughout the process. The absorbance of the samples was measured using UV-Vis spectrophotometer at wavelength of 470 nm from 0 min to 120 min at 15 minutes intervals.

Antioxidant activity (A_A) was determined as percent inhibition relative to control sample (without the extract):

A_A (%) = [($R_{control}$ – R_{sample}) / $R_{control}$] X100

Where $R_{control}$ and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively.

Degradation rates (R_D) were calculated according to first-order kinetics:

$R_D = \ln(A_t/A_x) \times 1/t$

where ln is natural log, A_t is the initial absorbance at 470 nm at t = 0 and A_x is the absorbance at 470 nm at t = 10, 20 and 30 min (Akowuah et al., 2004).

The oxidation rate ratio (R_{OR}) was calculated by

$R_{OR} = R_{sample}/R_{control}$

where R_{sampke} and $R_{control}$ are as described earlier.

The antioxidant activity coefficient (C_{AA}) was calculated using:

$C_{AA} = [(A_{s(120)} - A_{c(120)}/A_{(c)} - A_{c(120)})] \times 1000$

Where $A_{s(120)}$ is the absorbance of the sample containing antioxidant at t =120 min, $A_{c(120)}$ is the absorbance of the control at t = 120 min and $A_{(c)}$ = is the absorbance of the control at t = 0 min (Sonia and Mohamed, 2008).

Statistical analysis

Data are presented as means ± standard deviation. Statistical analysis was performed using SPSS software to analyze the statistical significance. The values $p<0.01$, $p<0.05$ considered as significant.

RESULTS AND DISCUSSION

HPLC analysis

Figure 1 illustrates the separation of QN and KMP standard and the separation of the markers from sample extract of MM leaves collected from Yan, Kedah. The linearity curve was checked by running a triplicate standard injection prior to injection of the MM sample from various locations. Recalibration was carried out if the mean difference between the known standard and the calculated values exceeds 5%.

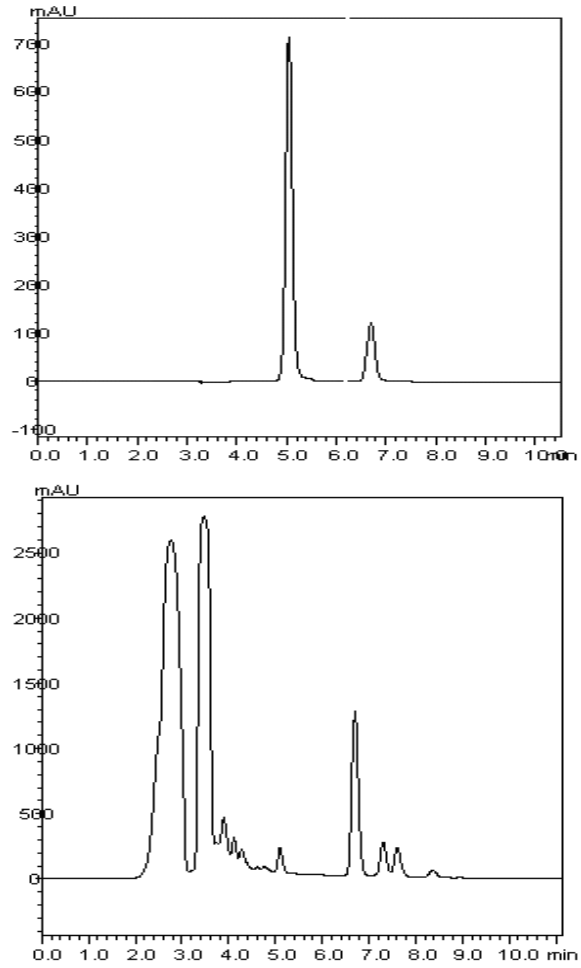

Fig. 1: Chromatogram of (a) QN and KMP standard mixture, (b) MM sample from Yan, Kedah where peak 1: quercetin and 2: kaempferol.

System suitability tests

Reproducibility, linearity and detection limit

A standard mixture solution containing of each 20 µg/mL of QN and KMP was analyzed five times to determine the reproducibility of the peak areas and retention time under the optimum conditions in this experiment. The relative standard deviations (R.S.D.) of the peak areas and retention time were 1.21 and 1.35 % for QN and 2.04 to 2.18 % for KMP respectively. A

series of the standard mixture solutions containing QN and KMP were tested to determine the linearity between the standard mixture concentration and peak areas. Good correlation of linearity has been achieved in the range of 1 – 100 μg/mL for both QN and KMP with the correlation coefficients for both the compounds were >0.9990. The detection limits were evaluated on the basis of a signal-to-noise ratio of 3 (S/N = 3), and found to be 0.10 μg/mL and 0. 20 μg/mL for QN and KMP respectively.

Precision and stability

The intra-day and inter-day precisions (expressed as the relative standard deviation (R.S.D.) for retention time and peak area were determined for both QN and KMP by repeated analysis (n = 5). The results shows (Table 1) that intra-day and inter-day relative standard deviations for retention time and for peak area are both quite low and the precision is good. For stability test, a sample solution (methanolic extract) was freshly prepared and the content of both QN and KMP was determined. The sample was then analyzed every day for one week and the sample solution was found to be stable in refrigeration condition for up to 2 weeks without any significant decrease in the concentration for both KMP and QN. However, both the standard sample in bench condition showed significant decrease in concentration just after 4 days of the test.

Table. 1: Intra-day and inter-day precision study.

Compound	Intra-day R.S.D. for retention time (%)	Intra-day R.S.D. for peak area (%)	Inter-day R.S.D. for retention time (%)	Inter-day R.S.D. for peak area (%)
QN	1.45	1.28	1.88	1.64
KMP	1.62	1.45	1.75	1.53

Recoveries of QN and KMP from MM leaves

The accuracy of the method was evaluated through recovery studies. The recovery experiment was performed at three concentrations of the standard added to sample solutions, in which the marker content had been determined, using sample from Bumbung Lima. The results for the recovery for QN and KMP were in the range 97.6 - 98.9% and 98.0 - 99.2% respectively with the RSD values less than 1%. The recoveries of QN and KMP are shown in table 2.

Table. 2: Recovery of QN and KMP from *Melastoma malabathricum* leaves (n=5).

Compound	Amount added (ng)	% Recovery	RSD (%)
QN	100	97.6	0.23
	500	98.5	0.17
	750	98.9	0.21
KMP	100	98.4	0.51
	500	99.2	0.31
	750	98.0	0.11

Quantification of QN and KMP in MM leaves collected from different locations in Malaysia

Low pressure isocratic RP-HPLC method was used for the identification and quantification of QN and KMP content in the MM leaves. In this analysis MM sample from 12 different locations in northern part of Malaysia were analyzed and their QN and KMP content were determined as shown in figure 2. The rare variety of MM with white flowers from Sungai Petani was also analyzed. The HPLC chromatographic profiles of the MM samples were qualitatively similar but the results showed considerable variations in the concentrations of the marker compounds. The results indicated that there were significant differences of QN and KMP contents between different sources of leaves. The content of KMP was found to be higher than QN in all the samples analysed.The highest percentage of QN content was shown by MM sample from Yan and the lowest was shown in Gurun. As for KMP, highest amount was noted from Sungai Petani (white petals) sample and the lowest from Kota Muda. The significant differences of yield content of QN and KMP between the different locations could be due to the locality and soil content. Based on the results obtained, the developed and validated HPLC method herein will be useful for the routine analysis of QN and KMP in MM sample for standardization, quality assurance work as well as for determination of adulteration of the crude drug.

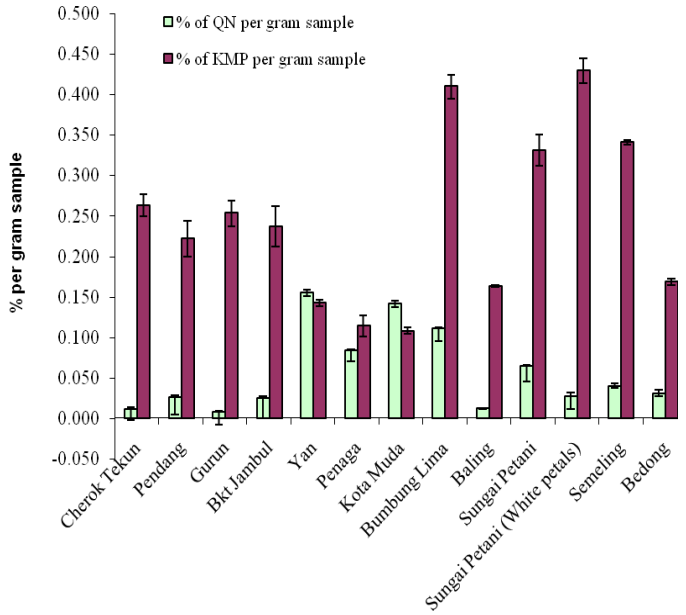

Fig. 2: Percent concentrations of marker quercetin (QN) and kaempferol (KMP) in leaf samples of MM collected from different locations. The values are presented as mean ± SD from triplicate determination. * indicates the significance level at $p < 0.01$ for QN, ** indicates the significance level at $p < 0.05$ for KMP compare with other location.

Antioxidant activity of MM extracts using β-carotene bleaching method

The antioxidant activities of the MM using 3 different solvent extracts were determined by the β-carotene-lineolate bleaching method. Linoleic acid produces hydroperoxides as free radicals during incubation at 50ºC. The hydroperoxides attack the β-carotene molecule as a result it undergoes a rapid discolouration. The corresponding decrease in absorbance can be monitored spectrophotometrically using UV-Vis spectrophotometer.

With the presence of antioxidative compounds in the extracts, the extent of β-carotene bleaching can be hindered by acting on the linoleate-free radical and other free radicals formed in the system. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts.

The absorbance decreased rapidly in sample extracts without antioxidant whereas, in the presence of an antioxidant, the colour of β-carotene and the absorbance value retained for a longer time. The absorbance of the emulsion decreased with time is shown in Figure 3. The 3 different solvent extracts, quercetin, the control (without the extract) and butylated hydroxytoluene (BHT) showed variation in anti-oxidant activity (Table 3). There was a correlation between degradation rate and the bleaching of β-carotene; where the extract with the lowest β-carotene degradation rate exhibited the highest antioxidant activity. Chloroform and methanol extracts shows higher antioxidant activity than synthetic antioxidant, BHT. Quercetin, one of the bioactive constituent of MM, which is known for its antioxidant activities, exhibited relatively high antioxidant activity. The antioxidant activity of MM extracts followed the order of chloroform extract > methanol extract > quercetin > BHT > ethyl acetate extract. The 3 different solvent extracts (methanolic extract, ethyl acetate extract and chloroform extract) of MM samples showed considerable variation in antioxidant activities, which could be due to soil fertility levels, age of the plants, and variation in sample sourcing. It also reported that the level of active constituents in plants may vary due to the sampling stage (Janna et al., 2006). The presence of polyphenols in MM which are known to have antioxidant properties (eg: quercetin, quercetrin, rutin, kaempferol and gallic acid) as reported earlier (Mohd. Joffry et al., 2011) and the varying polarity of these polyphenols could also contribute to the overall antioxidant pattern of the different solvent extract of MM. As such, we propose that proper sampling time and technique should be practiced when researchers intended to work on isolation and separation of active biomarkers from medicinal plants. Apart from that, consistency in sampling should be adhered with in order to obtain maximum output of the planned research.

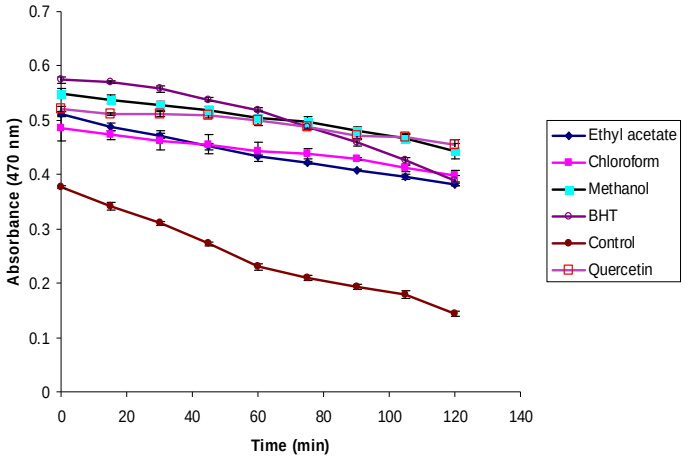

Fig. 3: Antioxidant activity of MM leaf extracts as assessed by β-carotene bleaching method.

Table. 3: Antioxidant activity of *Melastoma malabathricum.*

	Antioxidant ratio, R	Antioxidant activity (%AA)	Antioxidant activity coefficient, CAA
Ethyl acetate extract	0.5559	44.41	717
Chloroform extract	0.1672	83.28	1087
Methanol extract	0.1826	81.74	1715
Quercetin	0.3146	68.54	1050
BHT	0.3314	66.86	1478

CONCLUSION

In conclusions, we have developed a simple, rapid and reliable HPLC method for the quantification of bioactive markers from MM. The developed and validated HPLC method herein is the first HPLC method to be reported for the separation of bioactive flavonoids from MM. The method has been successfully applied for the determination of those markers in MM samples obtained from various locations in Malaysia. The method is currently being used in the pharmacokinetic studies involving MM extracts in our laboratory. Based on our findings, all the different solvent extracts of MM showed considerable variation in antioxidant activities and are comparable with those commercially available antioxidants thus making MM as one of the potential natural antioxidant remedy.

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