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ORIGINAL ARTICLE

A study on the total phenols content and antioxidant activity of essential oil and different solvent extracts of endemic plant *Merremia borneensis*

M. Amzad Hossain *, **Muhammad Dawood Shah**

Biotechnology Research Institute, Universiti Malaysia Sabah, Locked Bag No. 2073, 88999 Kotakinabalu, Sabah, Malaysia

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Abstract This study is planned to determine the antioxidant activity and total phenols content of the essential oil and different solvent extracts of the endemic plant *Merremia borneensis*. The antioxidant activities of the extracts were examined by three different methods, DPPH, β-carotene and reducing power assays. In all methods, aqueous ethanol extract exhibited a higher activity potential than that of other extracts (hexane, chloroform, ethyl acetate and butanol) and the essential oil. As assumed, the amount of total phenolics was very high in this extract. Chloroform extract has been found to be rich in flavonoids. A positive result was observed between the antioxidant activity potential and total flavonoid levels of the extracts.

1. Introduction

The processes of oxidation are intrinsic in the management of energy of all living organisms and are, therefore, kept under strict control by several cellular mechanisms (Halliwell and Gutteridge, 2007). However, the production excessive free radicals and the antioxidant protection due to unbalanced mechanisms result in the onset of numerous diseases and accelerate ageing. The antioxidants of low molecular weight are considered as possible protection agents reducing oxidative damage of the human body, when the internal enzymatic mechanisms fail or are inadequately efficient (Halliwell, 1995). Oxidation mediated by free radical reactions is also responsible for the rancidity of unpreserved food rich in unsaturated fatty acids and the natural antioxidants are suggested as a superior alternative for the synthetic ones such as BHA or BHT (Li et al., 2008). Therefore, there is a growing interest day by day in the substances exhibiting antioxidant properties, which are supplied to humans and animals as food components or as specific preventative pharmaceuticals (Sarikurkcu et al., 2009).

The plant kingdom is a good source to produce a wide range of natural antioxidants. However, still there is not enough knowledge and data about the practical usefulness of

* Corresponding author. Tel.: +60 0109571462; fax: +60 088320993.
E-mail address: dramzadh@gmail.com (M. Amzad Hossain).
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most of them. Groups of secondary plant metabolites, antioxidant phenolics, and flavonoids are commonly found in various fruits, vegetables and herbs and they have been shown to provide a fruitful defence against oxidative stress from oxidizing agents and free radicals (Matkowski, 2006; Sarikurkcu et al., 2009; Antolovich et al., 2000). Most of the herbal infusions, commonly used as home medicines have antioxidative and pharmacological properties related to the presence of phenolic compounds, especially phenolic acids derivatives and flavonoids. Polyphenols, such as phenolic derivatives and flavonoids are also known for their ability to prevent fatty acids from oxidative decay, and provide an additional value to plants used as food ingredients, rich for example in rosmarinic acid (Fecka et al., 2007).

Merremia borneensis is a shrub widely distributed in the South East Asia especially in Malaysia (Baytop, 1984). The leaves are suitable to be used as wrapper to the famous fermented rice or fermented tapioca known in Malaysia as 'Tapai.' The plant creeps well and is very productive in shady areas as well as in open areas and is known to blanket a whole tree or on any object that it chooses to make as its habitat. The stem contains latex that are higly sticky and the flowers are white in colour. This plant has been shown to have a wide range of biological activities. The leaves, according to natives in Sarawak, Malaysia, are used to relieve breast cancer (Patiño et al., 2001).

The aim of this work is to determine the total phenols content and antioxidative properties of the essential oil and the extracts of *M. borneensis* by DPPH, β-carotene/linoleic acid and reducing power assays. Additionally, total flavonoid contents of essential oil, hexane, chloroform, ethyl acetate, butanol and aqueous ethanol extracts have been determined.

2. Experimental

2.1. Materials

β-Carotene (99.99%), linoleic acid, α,α-diphenyl-β-picrylhadrazyl (DPPH) and butylated hydroxyanisole (BHA) were obtained from E. Merck (Germany). Solvents used for extraction were ethanol, hexane, butanol, chloroform (HPLC grade) obtained from Merck (Darmstadt, Germany). The deionised water was obtained from water distillation plants in our laboratory. All other chemicals were of analytical grade or GC grade. UV spectra UV–Visible spectra measurements were done using a Spectro (Thermo Fisher Scientific, model 4001/4) spectrophotometer.

2.2. Sample collection

The green leaves of *M. borneensis* were collected from the campus of University Malaysia Sabah, Malaysia. The plants were harvested during the month of September 19, 2010. The leaves were collected at 2:00 pm–3:00 pm on September 19, 2010 and packed in polyethylene bags and stored at 4 °C until required. Approximately 50 g of leaves were ground using a grinder (Blender 80115) for 20 s. The unfermented *M. borneensis* leaves were kept in the oven at 40 °C and put in a desiccator for at least 24 h prior to analysis.

2.3. Isolation of the essential oil

The air-dried leaves and stems (250 g for each) of *M. borneensis* were subjected to hydrodistillation for 3 h using a Clevenger type apparatus. The oil was dried with anhydrous sodium sulphate and preserved in a sealed vial at 4 °C until further analysis.

2.4. Extraction

The small pieces of the samples were homogenised in a grinder for 3 min to 40-mesh size. The air-dried leaves and stems of *M. borneensis* were pulverized into powdered form. The dried powder sample (50 g) was extracted three times with 70% ethanol (70% ethanol – 30% water) (3 × 200 mL) at room temperature and the solvents from the combined extracts were evaporated by a vacuum rotary evaporator (Buchi Labortech AG, model 1, R-215). The methanol extract was (7.3 g) suspended in water and extracted successively with hexane, chloroform, ethyl acetate and butanol to give hexane (1.97 g), chloroform (0.93 g), ethyl acetate (0.78 g) and butanol (0.391 g) and residual ethanol fractions (0.58 g), respectively. The extract was filtered through Whatman No. 41 filter paper to obtain particle free extract. The residue was reextracted twice and filtered. The extracts were concentrated and dried under vacuum. The same procedure was followed for the other solvents, such as hexane, ethyl acetate, chloroform and butanol for antioxidant fractions (Jena et al., 2002) and the extracts were used to determine their antioxidant activity. Solvents (analytical grade) for extraction were obtained from analytical reagent grade.

2.5. Determination of total phenols content

The level of total phenols in the crude extracts was determined by using Folin–Ciocalteu reagent and external calibration with gallic acid. Briefly; 0.2 mL of extract solution and 0.2 mL of Folin–Ciocalteu reagent were added and the contents mixed thoroughly (Singelton et al., 1999). After 4 min, 1 mL of 15% Na_2CO_3 was added, and then the mixture was allowed to stand for 2 h at normal temperature. The absorbance was measured at 760 nm using a Spectro (Thermo Fisher Scientific, model 4001/4) spectrophotometer. The concentration of the total phenolics was calculated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The determination of total phenolic compounds in the fractions was carried out in triplicate and the results were averaged.

2.6. Determination of total flavonoids

Total flavonoid contents of *M. borneensis* were determined by using the aluminium chloride colorimetric method as described by Willet (2002), with some modifications. Aqueous ethanol extracts (0.5 mL), 10% aluminium chloride (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (4.3 mL) were mixed. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm using a Spectro (Thermo Fisher Scientific, model 4001/4) spectrophotometer. Quercetin was used to make the calibration curve. The calculation of total flavonoids in the extracts was carried out in triplicate and the results were averaged.

2.7. Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of essential oil, hexane, ethyl acetate, chloroform, butanol and aqueous ethanol extracts of *M. borneensis* was evaluated by the method of Prieto et al. (1999). An aliquot mixture of 0.1 mL of extract sample solution (100 μg/mL) was mixed with 1 mL of mixture reagent solution (0.6 M sulphuric acid, 30 mM sodium phosphate and 4 mM ammonium molybdate). The sample tubes were sealed and incubated in a boiling water bath at 95 °C for 90 min. After incubation the reactant samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A typical blank sample contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as the rest of the samples. For samples of unknown composition, water soluble antioxidant activity was expressed as equivalents of ascorbic acid.

2.8. Antioxidant assay using β-carotene-linoleate model system

The antioxidant activity of the *M. borneensis* extracts were evaluated using β-carotene-linoleate model system as described by Jayaprakasha et al. (2001) with some modification. 0.1 mg of β-carotene in 0.2 mL of chloroform, 10 mg of linoleic acid and 100 mg of Tween-20 (polyoxyethylene sorbitan monopalmitate) were mixed. The solvent was removed at 40 °C under vacuum and the resulting mixture was diluted with 10 mL of water and was mixed well. To this mixture, 20 mL of oxygenated water was added. Four milliliter aliquots mixtures were pipetted into different test tubes containing 0.2 mL of extracts (50 and 100 ppm) and BHA (50 and 100 μg) in ethanol. BHA was used for comparative purposes. A control containing 0.2 mL of ethanol and 4 mL of the above emulsion was prepared. The tubes were placed at 50 °C in a water bath and the absorbance at 470 nm was taken at zero time ($t = 0$). The absorbance was continued to be measured until the colour of β-carotene disappeared in the control tubes ($t = 60$ min) at an interval of 15 min. A mixture prepared as mentioned above without β-carotene served as blank. All determinations were carried out in triplicate.

The antioxidant activity (AA) of the *M. borneensis* extracts was evaluated in terms of bleaching of the β-carotene using the following formula,

$$AA = 100[1 - (A_0 - A_t)/(A_0^0 - A_t^0)]$$

where A_0 and A_0^0 are the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A_t^0 are the absorbance of test sample and control, respectively, after incubation for 60 min. The results were expressed in % basis in preventing bleaching of β-carotene.

2.9. Radical scavenging activity using DPPH method

Radical scavenging activity of the *M. borneensis* extracts was determined essentially as described by Blois (1958) with some modification. The extracts of different concentrations (25, 50 and 100 μL equivalent to 25, 50 and 100 μg, respectively) and BHA (25, 50 and 100 μg) were taken in different test tubes. The volume was adjusted to 100 μL by adding MeOH. Five mililiter of 0.1 mM methanol solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27 °C for 20 min. The control was prepared as the same without any extract and MeOH. The changes in the absorbance of the prepared samples were measured at 517 nm. Radical scavenging activity was estimated as the inhibition percentage and was calculated using the following formula,

$$\text{Measurement of radical scavenging activity}(\%) = (\text{Control OD} - \text{sample OD}/\text{Control OD}) \times 100.$$

2.10. Statistical analyses

Experimental results were mean ± S.D. of three parallel measurements and analyzed by SPSS 10 (SPSS Inc. Chicago, IL). Differences between means were determined using Tukey multiple comparisons and least significant difference (LSD). Correlations were obtained by Pearson correlation coefficient in bivariate correlations. P values <0.05 were regarded significant.

3. Results

The yields of essential oil, hexane, ethyl acetate, chloroform, butanol and aqueous ethanol extracts of the leaves of *M. borneensis* were 1.9%, 3.1%, 12.98%, 21.90%, 10.00% and 32.58%, respectively. The total phenolic contents of the extracts and essential oil as determined by Folin–Ciocalteu method are reported as gallic acid equivalents (Singelton et al., 1999) (Table 1). Among the five extracts and essential oil, aqueous ethanol extract was containing the highest (30.35%) amount of phenolic compounds followed by chloroform extract (20.25 %), butanol extract (6.84%), ethyl acetate extract (6.58%) and hexane extract (5.90%). However, no phenolic compounds were observed in the essential oil. In recent studies, it has already been reported that the yield of extractable compounds was highest in aqueous ethanol extract from the peel and seeds of pomegranate in comparison with the solvents, such as chloroform, butanol, ethyl acetate and hexane (Negi et al., 2002).

The content of total phenolic was carried out based on the absorbance values of the various extract solutions, reacted with Folin–Ciocalteu reagent and compared with the standard solutions of gallic equivalents as described above (Singelton et al., 1999). Data obtained from the total phenolic method support the key role of phenolic compounds in free radical scavenging and/or reducing systems. As assumed, amount of the total phenolics was very high in aqueous ethanol extract

Table 1 Phenols content (as gallic acid equivalent) extracts of the leaves of *Merremia borneensis*.

Extract	Phenolics (% w/w)
Essential oil	–
Hexane extract	5.90 ± 0.13
Ethyl extract	6.58 ± 0.26
Chloroform extract	20.25 ± 0.32
Butanol extract	6.84 ± 0.49
Aqueous ethanol extract	30.35 ± 1.08

The values are means ± SD of three replicates.

Table 2 Total flavonoid content extracts of the leaves of *Merremia borneensis*.

Extract	Phenolics (% w/w)
Essential oil	–
Hexane extract	3.44 ± 0.21
Ethyl extract	24.51 ± 0.34
Chloroform extract	58.83 ± 0.44
Butanol extract	12.54 ± 1.22
Aqueous ethanol extract	53.28 ± 1.78

The values are means ± SD of three replicates.

Table 3 Antioxidant capacity of the leaves extracts of *Merremia borneensis* by phosphomolybdenum method.

Extract	Phenolics (% w/w)
Essential oil	25.31 ± 2.09
Hexane extract	16.22 ± 1.49
Ethyl extract	5.61 ± 0.92
Chloroform extract	28.52 ± 2.21
Butanol extract	13.92 ± 1.71
Aqueous ethanol extract	31.09 ± 9.1

The values are means ± SD of three replicates.

(30.35 ± 1.08) (Table 1). On the other hand, chloroform extract has been found to be rich in flavonoids with a value of 58.83 ± 0.44 mg quercetin/g. However, no flavonoid compounds were observed in the essential oil.

The way of determination of the level of total phenolics is not based on absolute measurements of the amounts of phenolic compounds, but is in fact based on their chemical reducing capacity relative to gallic acid. It is very important to point out that; there is a positive relationship between antioxidant activity potential and amount of phenolic compounds of the crude extracts. From the phenol antioxidant index, a combined measure of the quality and quantity of antioxidants in vegetables has been obtained (Elliot, 1999). In the present study the responses of the crude extracts in this assay may arise from the variety and/or quantity of phenolics found in five different extracts of the leaves of *M. borneensis*. Fruit and vegetables are the predominant sources of antioxidant vitamins (vitamin E, vitamin C, precursor of vitamin A i.e., β-carotene), which act as free radical scavengers, making these foods essential to human health (Elliot, 1999). However, more than 80% of the total antioxidant activity in fruits and vegetables comes from the ingredients other than antioxidant vitamins, indicating the presence of other potentially important antioxidants in these foods (Miller and Rice-Evans, 1997). The phenolic derivatives compounds are the vital antioxidants which exhibit scavenging efficiency on the free radicals; reactive oxygen species are numerous and widely distributed in the plant kingdom (Prior and Cao, 2000). In the present study, the relative antioxidant ability of the *M. borneensis* extracts and the essential oil was investigated through three in vitro models, such as antioxidant capacity by phosphomolybdenum method, β-carotene-linoleate system, and radical scavenging activity using, α, α-diphenyl-β-picrylhydrazyl (DPPH) method.

The result of total flavonoid contents of the five extracts of *M. borneensis* and essential oil is given in Table 2. The total flavonoid contents varied from 3.44 to 58.83 mg quercetin/g weight. Chloroform extract has been found to be rich in flavonoids. The variation may be due to environmental conditions and geographical distribution, which can modify the constituents of the plant.

Table 4 Antioxidant activity of extracts from the leaves of *Merremia borneensis* and BHA by β-carotene-linoleate model system (% inhibition of bleaching of β-carotene).

Extract/BHA	50 µg	100 µg
BHA	95.04 ± 0.02	97.53 ± 0.14
Hexane extract	21.62 ± 1.08	43.83 ± 0.48
Ethyl acetate extract	43.95 ± 0.42	55.38 ± 0.29
Chloroform extract	51.22 ± 0.54	61.42 ± 1.03
Butanol extract	32.33 ± 0.78	48.63 ± 0.92
Essential oil	45.68 ± 0.54	59.27 ± 0.44
Aqueous ethanol extract	62.48 ± 0.23	84.32 ± 0.19

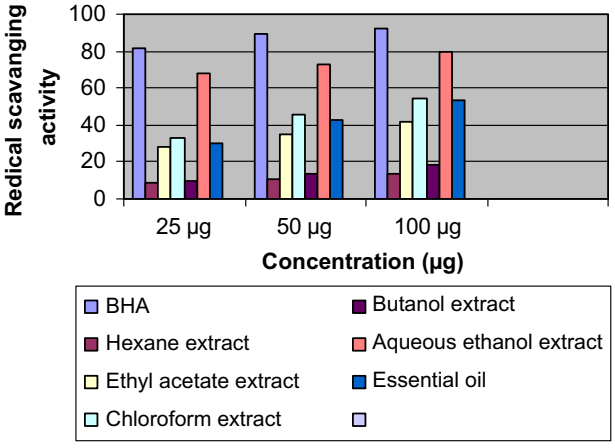

Figure 1 Redical scavanging activity of *Merremia boreneensis* of leaves extracts and essential oil by DPPH method.

The antioxidant capacity of the five extracts and essential oil was measured spectrophotometrically through phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of the extracts and essential oil of *M. borneensis* was found to decrease in the order, aqueous ethanol extract > chloroform > essential oil > hexane > butanol > ethyl acetate extract (Table 3).

4. Discussion

The antioxidant activity through β-carotene-linoleate system of the five extracts and essential oil of *M. borneensis* at 50 and 100 µg/mL concentrations was compared, respectively, with butylated hydroxyanisole and it is presented in (Table 4). The addition of the extracts of *M. borneensis* and butylated hydroxyanisole at 50 µg/mL concentrations prevented the bleaching of β-carotene to different degrees. β-Carotene in this method undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid formed free radical upon the abstraction of a hydrogen atom from one of its diallylic methylene groups,

which attacks the highly unsaturated β-carotene molecules. As a result, β-carotene was oxidised and broken down in parts, subsequently the system loses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically. In our present study, the five extracts from *M. borneensis* were found to hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. Aqueous ethanol extract, chloroform extract, essential oil, ethyl acetate extract, butanol extract and hexane extracts showed 84.32%, 61.42%, 59.27%, 55.38%, 48.63% and 43.83% antioxidant activity, respectively, at the concentration of 100 μg/ml.

The free radical scavenging activity of the leaves extracts and essential oil of *M. borneensis* were tested through DPPH method and the results are presented in the (Fig. 1). The role of antioxidants is their interaction depends on oxidative free radicals. The summary of DPPH method is that the antioxidants react with the stable free radical i.e., α,α-diphenyl-β-picrylhydrazyl (deep violet colour) and convert it to α,α-diphenyl-β-picrylhydrazine with discolouration. The discolouration indicates the scavenging potentials of the sample antioxidant such as phenolic compounds. In the present study the five extracts and essential oil of *M. borneensis* were able to decolourise DPPH and the free radical scavenging potentials of the extracts of were found to be in the order of aqueous ethanol extract > chloroform > essential oil > ethyl acetate extract > butanol > hexane extract. It has been found that hydrocarbon, cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (hydroquinone, pyrogallol, etc.), and aromatic amines, such as *p*-phenylene diamine, *p*-aminophenol etc., reduce and decolourise α,α-diphenyl-β-picrylhydrazyl by their hydrogen free radical scavenging ability (Blois, 1958). In our study may be it appears that the five extracts from the leaves of *M. borneensis* possess hydrogen donating capabilities to act as an antioxidant.

In our study, the order of decreasing antioxidant activity among the *M. borneensis* extracts through all the methods was found to be aqueous ethanol extract > chloroform extract > essential oil > ethyl acetate extract > butanol extract > hexane extract. This order is similar to the phenolic contents of the extracts that showed the extent of antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract (Abdille et al., 2005). In the present study it is found that the aqueous ethanol leaves extract of *M. borneensis* contains substantial amount of phenolics and it is the extent of phenolics present in this extract being responsible for its marked antioxidant activity as assayed through various in vitro models. Several reports have finally shown close relationship between total phenolic contents and antioxidative activity of the fruits, plants and vegetables (Deighton et al., 2000; Abdille et al., 2005; Vinson et al., 1998). The chemical composition and chemical structures of active extract components are important factors governing the efficacy of natural antioxidants, the antioxidant activity of an extract could not be explained on the basis of their phenolic content, which also needs their characterization (Heinonen et al., 1998). For instance, it has already been reported that phenolic compounds with ortho- and para-dihydroxylations or a hydroxy and a methoxy group or both are more effective than simple phenolics (Shahidi and Naczk, 2004). However, different types of actions of the phenolics present in the extracts cannot be ruled out. So far we know this is the first report that envisages the antioxidant activities of *M. borneensis* extracts. Hence the leaves of *M. borneensis* could be a good source of antioxidant phenolics. Further studies are needed for the isolation and identification of individual phenolic compounds and also in vivo studies are needed for better understanding of their mechanism of action as antioxidant.

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