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ORIGINAL ARTICLE

Total flavonoids content and biochemical screening of the leaves of tropical endemic medicinal plant *Merremia borneensis*

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Abstract The developing and under developed countries mostly rely on traditional medicines. This herbal or traditional medicine involves the use of different types of organic extracts or the bioactive chemical constituents. This type of biochemical investigation provides health care at an affordable cost. This survey such as ethnomedicine keenly represents one of the best avenues in searching new economic plants for medicines. Keeping this view in mind, the present study is carried out in *Merremia borneensis* leaves of University Malaysia Sabah, Sabah, Malaysia. The plant has several beneficial properties, such as antioxidant activity. The dry powder of the leaves of *M. borneensis* was extracted with hexane, ethyl acetate, chloroform, butanol and aqueous ethanol. The flavonoids content of the extracts was determined by Willet method. The flavonoids content of the extracts as quercetin equivalents was found to be highest in aqueous ethanol (53.28%) followed by chloroform (38.83%), ethyl acetate (24.51%), butanol (12.54%) and hexane extract (3.44%). The results suggest the presence of phytochemical properties in the leaves, which are used in curing the ailments.

1. Introduction

Phyto is the Greek word for plant. There are so many families of phytochemicals and they help the human body in a variety of ways. Phytochemicals may be protecting the human body from a host of diseases. Phytochemicals are non-nutritive plant bioactive chemicals that have protective or disease preventive properties. A plant produces these bioactive chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases. There are so many phytochemicals in fruits, vegetables and herbs and each works differently.

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In recent years, consumers desire to reduce the risk of or manage a specific health condition through improved diet. Plants have evolved different phytochemicals, ingredients and enzymes as an antioxidant defense to maintain growth and metabolism (Pandhair and Sekhon, 2006). Concern about improving health, involving agricultural products with high potential benefits, has enhanced advance research on antioxidants (Moore et al., 2005). Many degenerative human diseases including cancer, diabetics, cardio, and cerebro-vascular diseases have been recognized as being a possible consequence of free radical damage to lipids, proteins, and nucleic acids (Choi and Lee, 2009; Don et al., 1992; Gruber et al. 1999; Hoffman, 1975).

Various possible ways to fight these diseases is to improve our body's antioxidant defenses. Comparatively high consumption of vegetables and fruits has been associated with a lowered incidence of such degenerative and incurable diseases (Bajpai et al., 2009; Kalveram and Forck, 1978; Kirtikar and Basu, 1975; Kusumoto et al., 1995; Muruganandan et al., 2001). Fruits also help to improve health in other ways. For example, fruit juice, can also be taken to alleviate sore throat and seasickness. The functional bioactivity of a plant organic extract, in general, depends upon the presence of compounds, such as polyphenols, carotenoids, terpenoids, and chlorophyll (Negi et al., 2002). Plants can contribute in this area primarily due to the antioxidant activity of phenolic and flavonoids compounds (Higdon and Frei, 2003; Terao et al., 1994; Gardner et al., 2000; Hancock and Sahl, 2006; Li et al., 2001; Allan et al., 2004; O'Callaghan et al., 2004; Mhatre et al., 2009).

Several studies have revealed that the antioxidant activity may be from compounds, such as flavonoids, isoflavones, flavones, anthocyanins, catechins, and other phenolics (Kahkonen et al., 1999; Alothman et al., 2009; Isabelle et al., 2010). Oxidative stress has been linked to various curable and incurable diseases (Alothman et al., 2009; Isabelle et al., 2010), while food industry has long been concerned with issues, such as rancidity and oxidative spoilage of foodstuffs (Shahidi and Wanasundara, 1992). The enzymatic oxidation as well as auto oxidation of amino acid or lipids during storage and processing is the major reaction responsible for the deterioration in food quality affecting the colour, flavour, texture, and nutritive value of the foods. Antioxidants are often added to the foods to prevent the radical chain reactions of oxidation by inhibiting the initiation and propagation step leading to the termination of the reaction and a delay in the oxidation process.

Flavonoids, present as colouring pigments in plants also function as protective antioxidants at various levels. Some studies showed that flavonoids could protect membrane lipids from oxidation (Shahidi and Wanasundara, 1992). *Merremia borneensis* is a shrub widely distributed in the South East Asia especially in Malaysia. The leaves are suitable to be used as a wrapper for 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 any object that it chooses to make its habitat. The stem contains latex that is 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 (Prieto et al., 1999). *M. borneensis* is an important medicinal plant that is consumed in many parts of the world as herbal medicine. It has a high nutritive and oxidative value and is a rich source of vitamins A, B, and C besides several minerals such as calcium, phosphorus and iron. Though there are some reports on the antioxidant activities of apple fruits in relation to other fruits (Higdon and Frei, 2003; Terao et al., 1994; Gardner et al., 2000; Hancock and Sahl, 2006; Li et al., 2001), they only deal with one or two parameters and not in detail or do not suggest any possible components/mechanisms. During the course of our study on the biologically active constituents of this plant, we examined the constituents of the leaves of *M. borneensis* widely used in Sabah community, Malaysia. Hence, the aim of this present study has been made to investigate the phytochemical and biochemical screening of the powder leaves crude extract of *M. borneensis*.

2. Materials and methods

2.1. Materials

Aluminum chloride, quercetin, potassium acetate, hydrochloric acid, sulfuric acid were obtained from Sigma–Aldrich. Solvents for extraction were ethanol, hexane, butanol, chloroform (reagent grade) obtained from Merck (Darmstadt, Germany). The water was purified from water distillation plants in our laboratory. All other chemicals were of analytical grade or GC grade. UV spectra UV–Vis spectra measurements were done using a Spectro (Thermo Fisher Scientific, model 4001/4) spectrophotometer.

2.2. Sample collection

The fresh green leaves of *M. borneensis* were collected from the campus of University Malaysia Sabah, Malaysia. The leaves of this plant were harvested during the month of September, 2010. The leaves were collected from 2:00–3:00 pm on September 2, 2010 and packed in polyethylene bags and stored at 4 °C until required. The plant was initially identified by the morphological features and from the database present in the library, School of Biology, University Malaysia Sabah, Malaysia. 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. Extraction

The small pieces of leaves were homogenised in a grinder for 3 min to 30–40 mesh size. The air-dried leaves were pulverized into a powdered form. The dried leaves powder (50 g) was extracted three times with 70% ethanol (3×200 ml) at room temperature and combined. The combined extracts were evaporated by a vacuum rotary evaporator (Buchi Labortech AG, model 1, R-215). The ethanol extract was (7.3 g) diluted by 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 using Whatman No. 41 filter paper to obtain a particle free extract. The residue was re-extracted twice by solvent and filtered. The extracts were pooled and then concentrated and dried under vacuum pressure. The same extraction procedure was followed for the other solvents, such as hexane, ethyl

acetate, chloroform, and butanol for antioxidant fractions (Bhuiyan et al., 1996) and the extracts were used to explore their total flavonoids and other biochemical screening. Solvents (analytical grade) for extraction were obtained from E-Merck.

2.4. Determination of total flavonoids

Total flavonoids content of *M. borneensis* was determined by using the 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 content in the extracts was carried out in triplicate and the results were averaged.

2.5. Preliminary phytochemicals screening

One gram of the hexane, ethyl acetate, chloroform, butanol, and aqueous ethanol crude plant extracts of the powdered leaves of *M. borneensis* were dissolved in 100 ml of its own mother solvents to obtain a stock of concentration 1% (v/v). The obtained crude extracts were subjected to preliminary phytochemical screening following the methodology of Harborne (1998) and Kokate (2001).

2.6. Screening procedure

2.6.1. Test for alkaloids

Five milliliters of the stock crude extract was added to 2 ml of hydrochloric acid. One milliliter of Dragendroff's reagent was added to this acidic medium. An orange or red precipitation was immediately produced which indicates the presence of alkaloids.

2.6.2. Test for amino acids

To one milliliter of the crude stock extract was added a few drops of Ninhydrin reagent. The purple colour appearance shows the presence of amino acids.

2.6.3. Test for anthraquinones

Five milliliters of the crude stock extract solution was hydrolysed with diluted concentrated sulfuric acid extracted with benzene. Dilute ammonia solution was added to it. Appearance of rose pink colouration suggested the positive response for anthraquinones.

2.6.4. Test for flavonoids

To one milliliter of the crude stock extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour appeared in the plant crude extract, which became colourless on the addition of a few drops of dilute acid which indicates the presence of flavonoids.

2.6.5. Test for glycosides

The crude extract was hydrolysed by hydrochloric acid for few hours on a water bath. One milliliter of pyridine was added to the hydrolysate and a few drops of sodium nitroprusside solutions were added and then it was made alkaline with sodium hydroxide solution. The pink to red colour obtained shows the presence of glycosides.

2.6.6. Test for phytosterol

The plant crude extract was refluxed with a solution of alcoholic potassium hydroxide till complete saponification takes place. The whole mixture was diluted with water and extracted with ether. The ether layer was evaporated by water bath and the residue was tested for the presence of phytosterol. The residue was dissolved with a few drops of diluted acetic acid then 3 ml of acetic anhydride was added followed by a few drops of conc. H_2SO_4. The bluish green colour appeared which showed the presence of phytosterol.

2.6.7. Test for saponins

The crude extract stock solution was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 min. The formation of 1 cm foam layer showed the presence of saponins.

2.6.8. Test for steroids

One milliliter of the crude plant extracts was dissolved in 10 ml of chloroform and to it was added an equal volume of concentrated sulfuric acid from sides of the test tube. The upper layer turns into red and the sulfuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

2.6.9. Test for tannins

Three milliliters of the extract and a few drops of 1% lead acetate were added. A yellow precipitate was formed, indicates the presence of tannins.

2.6.10. Test for triterpenoids

Five milligrams of the crude plant extract was dissolved in 1 ml of chloroform and then 1 ml of acetic anhydride was added following the addition of 1 ml of conc. H_2SO_4. Formation of reddish violet colour indicates the presence of triterpenoids.

2.7. Statistical analyses

Experimental results were mean ± SD of three parallel measurements and analysed 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.

Table 1 Total flavonoids content extracts of the leaves of *Merremia borneensis*.

Extract	Total flavonoids (%, w/w)
Hexane extract	3.44 ± 0.21
Ethyl extract	24.51 ± 0.34
Chloroform extract	38.83 ± 0.44
Butanol extract	12.54 ± 1.22
Aqueous ethanol extract	53.28 ± 1.78

The values are means ± SD of three replicates.

Table 2 The analysis of biochemicals in the hexane, ethyl acetate, chloroform, butanol and aqueous ethanol extract of *Merremia borneensis*.

Biochemicals	Inference				
	Hexane	Ethyl acetate	Chloroform	Butanol	Aqueous ethanol
Alkaloids	–	+	+	+	+
Amino acids	–	+	+	+	+
Anthraquinones	–	–	–	–	–
Flavonoids	–	+	+	+	+
Glycosides	–	+	+	+	+
Phytosterol	+	–	–	–	+
Saponins	–	+	+	+	+
Tannins	–	+	+	+	+
Triterpenoids	+	+	+	+	+
Steroids	+	+	+	+	+

+ = presence; – = absence.

3. Results and discussion

The extraction yields of hexane, ethyl acetate, chloroform, butanol, and aqueous ethanol extracts of the powdered leaves of *M. borneensis* were 2.9%, 13.08%, 21.5%, 9.28%, and 30.3%, respectively. The total flavonoids content of the crude plant extracts as determined by Willet method are reported as quercetin equivalents (Table 1). Among the extracts, aqueous ethanol extract was containing the highest (53.28%) amount of flavonoids content compounds followed by chloroform (38.83%), ethyl acetate (24.51%), butanol (12.54%), and hexane extract (3.44%) (Table 1). In our previous studies, it has been reported that the yield of extractable compounds was the 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. Furthermore, the extraction of phenolic content from the fruit is commonly achieved with methanol or aqueous ethanol.

The result obtained in the present investigation (Table 2), the ethyl acetate, chloroform, butanol, aqueous ethanol, and methanol extracts of the powdered leaves of *M. borneensis* showed the presence of alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins, and triterpenoids. Further, ethyl acetate, chloroform and methanol extracts of the seeds showed the absence of anthraquinones.

Various herbs and herbal extracts contain different phytochemicals with biological activity that can show valuable therapeutic index. Most of the protective effects of fruits and vegetables have been attributed to active phytochemicals, which are the non-nutrient plant compounds. Different active phytochemicals have been found to possess a wide range of activities, which may help in the protection against incurable diseases. Biochemicals and phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids, and alkaloids have antiinflammatory effects (Liu, 2003; Manach et al., 1996; Latha et al., 1998; Akindele and Adeyemi, 2007; Orhan et al., 2007; Muruganandan et al., 2001; Nadkarni, 1954). Some polycyclic glycosides, flavonoids, tannins, and alkaloids have hypoglycemic activities (Oliver, 1980; Cherian and Augusti, 1995). Recently Rupasinghe et al. (2003) have reported that saponins possess hypocholesterolemic and antidiabetic properties. The mono, di and triterpenoids have also been shown to decrease blood sugar level in animal studies (Luo et al., 1999). High molecular weight steroids and triterpenoids showed analgesic properties (Sayyah et al., 2004; Malairajan et al., 2006). The steroids and saponins are also responsible for central nervous system activities (Argal and Pathak, 2006).

Biochemical and phytochemicals screening of the hexane, ethyl acetate, chloroform, butanol, aqueous ethanol. and methanol extracts of *M. borneensis* powder leaves used in this present study revealed that the crude extracts contained alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins, and triterpenoids (Table 2).

This study is only a preliminary study of the occurrence of certain properties of *M. borneensis* leaves an in-depth study will provide a good concrete base for all the biochemical and phytochemical functions mentioned above.

4. Conclusion

In this present study, we have found that biologically active biochemicals and phytochemicals were present in the ethyl acetate, chloroform, butanol, and ethanol extracts of *M. borneensis* powdered leaves. The medicinal properties of *M. borneensis* leaves crude extracts may be due to the presence of the above-mentioned active biochemicals and phytochemicals. Further studies are in progress in our laboratory to isolate the active components from the leaves of *M. borneensis*.

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