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In vitro total phenolics, flavonoids contents and antioxidant activity of essential oil, various organic extracts from the leaves of tropical medicinal plant *Tetrastigma* from Sabah

M Amzad Hossain*, Muhammad Dawood Shah, Charles Gnanaraj, Muhammad Iqbal

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

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ABSTRACT

Objective: To detect the *in vitro* total phenolics, flavonoids contents and antioxidant activity of essential oil, various organic extracts from the leaves of tropical medicinal plant *Tetrastigma* from Sabah. **Methods:** The dry powder leaves of *Tetrastigma* were extracted with different organic solvent such as hexane, ethyl acetate, chloroform, butanol and aqueous methanol. The total phenolic and total flavonoids contents of the essential oil and various organic extracts such as hexane, ethyl acetate, chloroform, butanol and aqueous ethanol were determined by Folin – Ciocalteu method and the assayed antioxidant activity was determined in vitro models such as antioxidant capacity by radical scavenging activity using α, α –diphenyl– β –picrylhydrazyl (DPPH) method. **Results:** The total phenolic contents of the essential oil and different extracts as gallic acid equivalents were found to be highest in methanol extract (386.22 mg/g) followed by ethyl acetate (190.89 mg/g), chloroform (175.89 mg/g), hexane (173.44 mg/g), and butanol extract (131.72 mg/g) and the phenolic contents not detected in essential oil. The antioxidant capacity of the essential oil and different extracts as ascorbic acid standard was in the order of methanol extract > ethyl acetate extract >chloroform> butanol > hexane extract also the antioxidant activity was not detected in essential oil. **Conclusions:** The findings show that the extent of antioxidant activity of the essential oil and all extracts are in accordance with the amount of phenolics present in that extract. Leaves of *Tetrastigma* being rich in phenolics may provide a good source of antioxidant.

1. Introduction

In current years, consumers look to reduce the risk or manage a specific health condition through improved food diet. Plants and fruits have envolved different phytochemicals and enzymes as antioxidant defense to maintain growth and metabolism system[1]. Concern about health improvement, involving agricultural products with their potential benefits, has enhanced research on antioxidants activity[2]. Many uncurable human diseases including cancer, cardio and cerebro-vascular diseases have been recognized as being a possible consequence of free radical damage to lipids, proteins, flavonoids and nucleic acids[3].

The possible ways to fight these uncurable diseases is to improve our body's transformation due to antioxidant defenses. High consumption of plants, fruits and vegetables has been associated with a lowered incidence of such degenerative or uncruable diseases[4]. Fruits also help to improve health in other ways. Fruit juice, vegetables juice for example, can alleviate sore throat and seasickness. The functional bioactivity of a plant extracts, essential oil in general, depends on the presence of compounds such as polyphenols, carotenoids and chlorophyll[5]. Plants can contribute in this area primarily due to the antioxidant activity of polyphenolic compounds[6].

Flavonoids and terpenoids mainly present as colouring pigments in plants also function as potent antioxidants at various levels. Some reports showed that flavonoids could protect membrane lipids from oxidation[7]. *Tetrastigma* is an important tropical medicinal plant that is consumed in many parts of the world as herbal medicine. It has a very high nutritive value and is a rich source of flavonoids,

*Corresponding author: M Amzad Hossain, Biotechnology Research Institute, Universiti Malaysia Sabah, Locked Bag No. 2073, 88999 Kota Kinabalu, Sabah, Malaysia.
Tel: + 237 5218 63/75390318
E-mail: waboponejosue@yahoo.fr

vitamins A, B and C besides several minerals such as calcium, phosphorus and iron. Though there are some studies on the antioxidant activities of apple and other fruits[8], they deal with some parameters but probable components/mechanisms remains unclear.

It is known that plants as well as extracts protect themselves against pathogens by various defense responses which include the production of antimicrobial peptides. Some antimicrobial ingredients are short cationic amphiphilic peptides form some important compounds of immune defenses[9]. The antimicrobial agent 'magainin' gene MSI-99 was isolated from skin secretions of the African clawed frog (Xenopus laevis), now is known to confer resistance against some broad spectrum of fungal and bacterial pathogens. Currently, a few studies have been made available on the expression of different analogues of magainin in tobacco, tomato, and potato[10] for obtaining incresed disease resistance. On the other hand synthetic substitution analogue of 'magainin', MSI-168, was used to transform leaf bases of apple in order to instill in-built resistance against microbial infections. Transgenic plants of *Tetrastigma* were thus produced by using the *Agrobacterium* strain EHA 105 harboring the plasmid pMSI-168 for transformation[11]. The successful expression of this synthetic peptide in *Tetrastigma* was obtained resulting in normal growth and fruiting of transgenic plants. Alteration in the genetic makeup could introduce changes in the biochemical processes of the plant extract, mainly the antioxidant activity makeup. To rule out this opportunity we sought to study the possible changes in the antioxidant status of various parts of transformed *Tetrastigma* extracts in comparison with non-transformed ones.

Several reports have revealed that the majority of the antioxidant activity may be from biochemicals such as flavonoids, isoflavones, flavones, anthocyanins, catechins and other phenolics[12-13]. Free oxidative stress has been linked to various uncruable diseases[12] while the food industry has been concerned with these issues such as rancidity and oxidative spoilage of foodstuffs[12]. The plant enzymatic oxidation as well as auto oxidation of 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. Natural antioxidants are often added to 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.

Tetrastigma sp., belongs to the grape family, Vitaceae[14]. It is also known as Lipoi, by the local communities in Sabah. These plants are vines that climb with tendrils and have compound leaves. *Tetrastigma* sp. is found only in subtropical and tropical regions of Asia, Malaysia, and Australia, where they grow in primary undisturbed rainforest. Species of these plants are available as being the sole hosts of parasitic plants in the family Rafflesiaceae, one of which, *Rafflesia arnoldii*, produces the largest flower in the world[15].

This plant species is considered to have medicinal properties as claimed by the locals in Sabah, around the area of *Tambunan* and the local ethnic Murut, that it is used to treat swelling pancreas, fever, cough and febrifuges. The leaves or shoots are boiled in water and the decoctions are consumed as medication[15]. It is also used for improving blood circulation, anti-inflammatory, analgesic, treatment of high fever, infantile febrile convulsion, pneumonia, asthma, hepatitis, rheumatism, menstrual disorders, sore throat, scrofula, improving the immune system and for anti-cancer[16-17].

However, synthetic antioxidants which are commonly used such as butylated hydroxyanisole (BHA) and butylated hydroxy toluene (BHT) are restricted by legislative rules because they are suspected to have some toxic effects and as possible carcinogens[16]. Keeping in this view, there has been a considerable interest by the industry and a growing trend in consumer preferences for natural antioxidants over synthetic compounds and elimination of synthetic antioxidants in food applications has given more impetus to explore natural source of antioxidants. Thus, antioxidants are of interest to both food and health scientists and there has been a convergence of interest among researchers in these fields as the role of antioxidants in the diet and their impact on human health has come under attention. In the present study, the antioxidant activity of the extracts of *Tetrastigma* was assayed through various in vitro models. So far we know, this is the first report on total phenolic, total flavonoids and antioxidant activity of leaves of Tetrastigma from Sabah.

2. Materials and methods

2.1. Chemicals

α, α-diphenyl-β-picrylhadrazyl (DPPH), gallic acid, BHA were obtained from E. Merck (Germany). Extraction solvents used for were ethanol, hexane, butanol, chloroform (HPLC grade) obtained from Merck (Darmstadt, Germany). 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 HITACHI, U-2000 spectrophotometer.

2.2. Sample collection

The green leaves of *Tetrastigma* were collected from Sabah hilly areas, Malaysia. *Tetrastigma* were harvested during the month of February, 2011. The leaves of this plant were collected at afternoon around 2:00 to 3:00 pm on February 17, 2011 and the leaves packed in polyethylene bags and stored at 4 ℃ until required. The plant initially identified by morphological features and data base present in the library, Borneo herbarium, University Malaysia Sabah, Malaysia and a voucher specimen has been deposited at the Forest Research Institute, Malaysia (FRIM) with voucher number

1113. Approximately 100 g of leaves were ground using a Braun grinder for 20 s. The unfermented *Tetrastigma* leaves were kept in the oven at 40 ℃ 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 40-mesh size. The air-dried leaves and stems of *Tetrastigma* were pulverized into powdered form by using blander. The dried leaves powder (50 g) was extracted with methanol (3×200 mL) at room temperature and the solvents were evaporated by a vacuum rotary evaporator (EYELA N- 1000, Japan). The methanol about extract was (7.3 g) suspended in distill water (100 mL) and extracted followed 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 methanol fractions (0.58 g), respectively. All the crude extracts were filtered through Whatman No. 41 filter paper to obtain particle free extract. The residue was again reextracted twice and filtered. The combined extracts were concentrated and dried under vacuum. The same experimental procedure was followed for the other solvents such as hexane, ethyl acetate, chloroform and butanol for antioxidant fractions[9] and the extracts were used to explore their phenolics and antioxidant activity. Solvents (analytical grade) for extraction were obtained from analytical grade.

2.4. Extraction of essential oil

The dried *Tetrastigma* leaves (50 g) were subjected to hydro distillation for 6 h using a distillation type apparatus. The essential oils from the leaves were dried over anhydrous sodium sulphate and further extracted with diethyl ether and preserved in a sealed vial at 4 ℃ until further analysis.

2.5. Determination of total phenolics

The concentration of total phenolics in the essential oil and different organic extracts were determined by using Folin-Ciocalteu reagent and external calibration with gallic acid. Briefly; about 0.2 mL of essential oil and extract solution and 0.2 mL of Folin-Ciocalteu reagent were added and the contents mixed vigorously. After shaking 4 min, 1 mL of 15% Na_2CO_3 was added, and finally the mixture was allowed to stand for 2 hours at room temperature. The absorbance was measured at 760 nm using HITACHI, U-2000 spectrophotometer. The concentration of the total phenolics was estimated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The quantification of phenolic compounds in all the fractions were carried out in triplicate and the results were averaged.

2.6. Determination of total flavonoids

Total flavonoid contents of *Tetrastigma* were estimated by using the aluminium chloride colorimetric method as described by Willet, with some modifications. Essential oil, methanol extract and it diverse extracts each separately (0.5 mL) mixed with 10% aluminium chloride (0.1mL), 1M potassium acetate (0.1mL) and distilled water (4.3 mL). At room temperature it was incubated for 30 min. The absorbance was measured at 415 nm using HITACHI, U-2000 spectrophotometer. Quercetin was used to make the calibration curve. The determination of total flavonoids in the essential oil and all extracts was carried out in triplicate and the results were averaged.

2.7. Radical scavenging activity using DPPH method

Radical scavenging activity of the extracted essential oil and all extracts was determined essentially as described by Blois with some modification. Different concentrations (25, 50 and 100 μL equivalent to 25, 50 and 100 μg, respectively) of the oil and extracts and BHA (25, 50 and 100 μg) were taken in different test tubes. The volume of all test tube was adjusted to 100 μL by adding MeOH. 0.1 mM methanolic solution (5 mL) of DPPH was added to these tubes and then shaken vigorously. The tubes were kepted to stand at 27 ℃ for 20 min. The control sample was also prepared as above without any extract and MeOH was used for the baseline correction. The changes in the absorbance were measured at 517 nm. Radical scavenging activity was calculated as the inhibition percentage and was calculated using the following formula:

% radical scavenging activity = (Control OD - sample OD/ Control OD) × 100.

2.8. Statistical analyses

Experimental results were expressed as mean ± SD 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 hexane, ethyl acetate, chloroform, butanol and methanol extracts of the leaves of *Tetrastigma* were 1.80%, 9.40%, 23.08%, 5.28% and 24.3%, respectively. The total phenolic contents of the essential oil and all extracts as determined by Folin-Ciocalteu method are reported as gallic acid equivalents (Table 1). Among the five extracts, methanol extract showed the highest [(386.22 ± 13.00) mg/g)] amount of phenolic compounds followed by ethyl acetate extract [(190.89 ± 11.97) mg/g], chloroform extract [(175.89 ± 19.45) mg/g], hexane extract [(173.44 ± 1.20) mg/g]

Table 1

Total phenolic and flavonoids content of various extracts of the leaves of *Tetrastigma*.

Extracts	Total phenolic(gallic acid equivalent)(% w/w)	Total flavonoids(% w/w)
Essential oil	–	–
Hexane extract	173.44±1.20	17.14±0.38
Ethyl acetate extract	190.89±11.97	41.13±0.27
Chloroform extract	175.89±19.45	51.68±0.51
Butanol extract	131.72±6.04	42.93±0.43
Methanol extract	386.22±13.00	72.01±1.65

The values are means±SD of three replicates.

and butanol extract [(131.72 ± 6.04) mg/g], but phenolic compounds was not detected in essential oil.

The result of total flavonoid contents of the essential oil and its five extracts of *Tetrastigma* is given in Table 1. The total flavonoid contents varied from 17.14 mg/g to 72.01 mg quercetin/g weight. It may be due to the variation of environmental conditions, which can modify the constituents inside of the plant.

Free radical scavenging activity (DPPH) of the leaves extracts and essential oil of *Tetrastigma* were tested using DPPH method and the results are presented in the (Figure 1). The essential oil did not show any activity with DPPH.

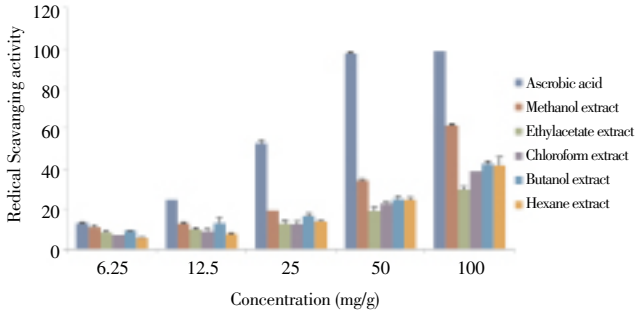

Figure 1. Redical scavanging activity of *Tetrastigma* of leaves extracts and essential oil by DPPH method.

4. Discussion

In recent reports, it has been reported that the yield of extractable compounds was highest in methanol 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 compounds from the fruits or vegetables is commonly achieved with methanol.

The total phenolics levels determined in this way are not absolute measurements of the amounts of phenolic compounds, but are in fact based on their chemical reducing capacity relative to gallic acid. It has been shown that the phenol antioxidant index, a combined measure of the quality and quantity of antioxidants in vegetables, fruits and plants. In the current study the responses of the extracts in this assay may arise from the variety and/or quantity of phenolics found in five different extracts of the leaves of *Tetrastigma*. Fruit, plants and vegetables are the main 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. Therefore, more than 80% of the total antioxidant capacity in fruits and vegetables comes from the ingredients other than antioxidant vitamins, indicating the presence of other potentially important antioxidants in these foods. The phenolic compounds such as flavonoids, terpenoids are the dominant antioxidants that exhibit scavenging efficiency on free radicals and reactive oxygen species are numerous and widely distributed in the plant kingdom. In the present study, the relative antioxidant ability of the *Tetrastigma* extracts was investigated through in vitro models such as antioxidant capacity by radical scavenging activity using α, α-diphenyl-β-picrylhydrazyl (DPPH) method.

The vital role of antioxidants is their interaction with oxidative free radicals. The assumption 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 degree indicates the scavenging potentials of the sample antioxidant. In this study, the five organic extracts of *Tetrastigma* 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> ethyl acetate extract >butanol>hexane extract. But the essential oil did not show any activity with DPPH. It has been showed that all chemical constituents such as tocopherol, polyhydroxy aromatic compounds (hydroquinone, pyrogallol, etc.), cysteine, glutathione, ascorbic acid, and also amines such as p-phenylene diamine, p-aminophenol etc. decolourise the α, α-diphenyl-β-picrylhydrazyl by their hydrogen donating ability. It appears that the five extracts from the leaves of *Tetrastigma* possess hydrogen donating capabilities to act as antioxidant.

In this study, the antioxidant activity decreasing in order among the *Tetrastigma* extracts assayed through the DPPH method was found to be methanol extract > ethyl acetate extract >chloroform> butanol > hexane extract. Similar order 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. In

this present study it is found that the aqueous methanol leave extract of *Tetrastigma* 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.

Many studies have conclusively shown close relationship between total phenolic contents and antioxidative activity of the fruits, plants and vegetables. The chemical composition inside the active extract, chemical constituents 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[5]. For that, it has been reported that phenolic compounds with ortho- and para-dihydroxylation or a hydroxy and a methoxy group are more effective than simple phenolics. Therefore, synergistic or additive 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 *Tetrastigma* extracts. Hence the leaves of *Tetrastigma* could be a good source of antioxidant phenolics. Further studies are materialized for the isolation and identification of individual phenolic compounds and also in vivo studies are needed for better understanding their mechanism of action as antioxidant

Conflict of interest statement

We declare that we have no conflict of interest.

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