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Evaluation of bitter melon (*Momordica charantia*) extract administration in the antioxidant and free radical scavenging activities of plasma and liver in male rat

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Abstract

The aim of this study was to determine the antioxidant activities of dietary bitter melon fruit supplemented in male rat model. In this study, three common tests for measuring antioxidant activity of the bitter melon were evaluated using 2, 2-diphenyl-l-picrylhydrazyl assay (DPPH assay), total phenolic acid assay (TPC assay) and Ferric reducing ability of plasma assa. (FRAP assay). Results showed that the extracting solvent significantly ($P<0.05$) altered the antioxidant property estimations of bitter melon fruit. Pure solvents were inefficient extraction media for antioxidant. Enhanced extraction yields were obtained from solvent containing higher water concentrations and 50% acetone is a recommended solvent for extracting antioxidants compounds from bitter melon fruit. High levels of antioxidant activities were detected in plasma and liver in treatment group compared to the control group, indicating that the fruit may serve as an excellent dietary source of natural antioxidants. The correlation between total phenolics and the antioxidant activities of bitter melon showed significant positive correlations (r2). These findings support the notion that the bitter melon's a good source of bioactive compounds. From the study it was observed that there was a significantly increased ($p \leq 0.05$) in plasma total antioxidant capacity in rat after the consumption of bitter melon.

Introduction

Bitter melon (*Momordica charantia*), a vegetable indigenous to tropical regions of Asia, belongs to the *Cucurbitaceous* family, contains an array of biologically active photochemical. These include triterpenes, proteins and steroids. Fruit and seeds of bitter melon are traditionally used as medicinal herbs as, anti-HIV, anti-ulcer, anti-inflammatory, anti-leukemic, antimicrobial, anti-diabetic, and anti-tumor, to name a few (Marderosiam, 2001). Free radicals are known to be the major cause of various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer (Slater, 1984; Cheng *et al*., 2003). Reactive oxygen species (ROS) include free radicals such as $dO_{2\_}$ (superoxide anion), dOH (hydroxyl radical), H_2O_2 (hydrogen peroxide) and $1O_2$ (singlet oxygen) can cause cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes (Compori, 1985; Halliwell, 1997). The tissue injury caused by ROS may include DNA damage (Halliwell and Gutteridge, 1984; Halliwell, 1997), protein damage (Bartold *et al*., 1984), and oxidation of important enzymes (Varani *et al*., 1985) in the human body. These events could consequently lead to the occurrence of various free radical related diseases. Recently, natural foods and food derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention, because they are known to function as chemopreventive agents against oxidative damage.

Many *in vivo* clinical studies have demonstrated the relatively low toxicity of all parts of the bitter melon plant when ingested orally. However, toxicity and even death in laboratory animals has been reported when extracts are injected intravenously or intraperitoneally (with the fruit and seed demonstrating greater toxicity than the leaf or aerial parts of the plant) (Sharma *et al*., 1960). Other studies have shown ethanol and water extracts of the fruit and leaf (ingested orally) to be safe during pregnancy (Sathish *et al*., 2010) The seeds, however, have demonstrated the ability to induce abortions in rats and mice, and

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the root has been documented with a uterine stimulant effect in animals (Naseem *et al*., 1998) .The fruits and leaves of bitter melon has demonstrated an *in vivo* antifertility effect in female animals(Bhakuni *et al*., 1989) in male animals, it was reported to affect the production of sperm negatively (Girini *et al*., 2005).Thus the objective of the present this work is to evaluate the antioxidant activities of commonly consumed fresh bitter melon (*Momordica charantia*) in Malaysia in relation to their total phenolic contents and free radical scavenging activities were also determined in the plasma and liver on male rat .

Materials and Methods

Chemicals

Folin–Ciocalteu phenol reagent, ferric chloride ($FeCl_3$•$6H_2O$), and HCl were obtained from Merck (Darmstadt, Germany) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2- pyridyl)-s-triazine (TPTZ), gallic acid and Trolox, and sodium acetate trihydrate were purchased from Sigma (USA). Sodium carbonate was purchased from RDH (Germany) while glacial acetic acid was from Mallinckrodt Baker (USA). All chemicals and reagents used in the study were of analytical grade.

Sample collection and preparation of M. charantia fruit aqueous extract

The fruits of *M. charantia* (MC) were obtained from the market in Kajang, Malaysia. The fruits of bitter melon were cleaned and cut into small pieces, and then oven dried at 60°C for 24 h. The dried sample was then pulverized using a mechanical grinder and passed through a 250 µm mesh and then stored at 4°C until use. The different types of solvent used were absolute methanol, ethanol, acetone, water and their aqueous solutions at 50%, 70%, and 100% concentrations. All tests were performed at room temperature.

Experimental animals

Studies were carried out using fourteen Sprague Dawley male rats each weighing between 150-200g. They were obtained from the animal house of the Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The animals were acclimatized to laboratory condition for a week before commencement of the experiment. Rats were fed ad libitum with commercial rat's food containing 10% MC fruit powder. The diets were prepared daily to minimize rancidity and oxidative damage, the rats were fed once daily and the left over feeds were collected before new feeding. After two months of feeding trial, the animals were fasted overnight and the day after were killed and the liver from each animal was collected and dissected and a 20% homogenate was prepared in ice cold phosphate buffer, pH 7.4 and centrifuged at 3,000 rpm for 15 min in a refrigerated centrifuge and then the supernatants were collected and stored at -8°C until analyzed.

Determination of the total phenolic contents:

Total phenolic content was determined according to the method of Hung *et al*. (2002). The total phenolic content is determined using the Folin-Ciocalteu reagent. The phenolic compounds are oxidized to phenolates by the reagent at alkaline pH in a saturated solution of sodium carbonate resulting in a blue molybdenum-tungstate complex. About 0.5 mL of Folin-Ciocalteau (10%, w/v,) is added to 0.1 mL sample, followed by the addition of 1mL of aqueous Na_2CO_3 (7.5%, w/v). The mixture was allowed to stand in the dark for 2 hours. The absorbance of the blue colour solution is read at 765 nm on a UV visible spectrophotometer (EPOCH) against blank (distilled water). Total phenolic concentration (mg mL^{-1}) of the sample was extrapolated from a standard curve, constructed using Gallic acid as a standard.

The 2, 2-diphenyl-l-picrylhydrazyl assay (DPPH) aAssay

The 2, 2-diphenyl-l-picrylhydrazyl assay (DPPH) is dissolved in 100% ethanol to 200 µM and sonicated for 5 min to obtain the stable free radical DPPH. The test compound is diluted in the ratio 1:1 with the DPPH. Solution in a 96-well micro plate. Appropriate controls were run in each series. Fresh DPPH.solution was prepared daily. Each fruit extract (100 µl) were to react with 3000 µl of the DPPH solution for 24 h in the dark condition. The standard curve was linear between 25 and 800 µM methanol. The fruit extract is tested in triplicate at different extraction solvents, such that a 50% fall in absorbance of the DPPH. The absorbance of the reaction mixture is measured after 25 min using a micro plate spectrophotometer (517 nm).

The percentage of DPPH scavenging activity was calculated using the following equation:

$$\text{Radical scavenging (\%)} = [(A_0 - A_1 / A_0) \times 100]$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample extracts

Determination of ferric reducing antioxidant power (FRAP)

The antioxidant capacity of each sample was estimated according to adapted procedure from Benzie and Strain (1996).The FRAP reagent was

prepared as follows : 300mM acetate buffer, pH 3.6 (3.1 gm sodium acetate trihydrate, plus 16 ml glacial acetic acid made up to 1 liter distilled water); 10 mμM TPTZ (2,4,6-tri (22-pyridyI)-s-triazine),in 40 mM HCL ; and 20 mM Fecl3 $6H_2O$ in ratio 10:1:1 to give the working reagent. FRAP reagent (3950 µL) was prepared and warmed at 37^0C, was mixed with 50 µL test sample reaction was monitored up to 30 min Fruit extracts (100 µL) were allowed to react with 1000 µL of the FRAP solution for 30 min in the dark condition .The mixture was transferred to micro plate plastic and its absorbance recorded at 595 nm on the spectrophotometer (EPOCH) Absorbance of a blank containing 50 µL of methanol with FRAP regent was recorded. Change in absorbance of each sample was computed. Furthermore, a known antioxidant, gallic acid, was used as standard to express FRAP value of the extracts. Known concentration of gallic acid, were used to prepare a standard curve with linear regression, which was used as reference for comparing the extracts. The result was expressed as milligrams of gallic acid, equivalents per 100 g of fresh sample (mg TE/g of FW).

Statistical analysis

The results were expressed as mean ± standard error (n = 7). The data were analyzed statistically by one way ANOVA and different group means were compared by Duncan's multiple range test (DMRT). The correlation between total phenolic content and antioxidant activity was established using a regression analysis at a 95% significance level. For all analyses, P values ≤ 0.05 were considered significant.

Result

The results showed (Table 1) that TPC, DPPH, and FRAP values were sensitive to extraction solvents whereby in pure solvents, water extract of MC fruit yield the highest extraction efficiency followed by acetone, ethanol and methanol, respectively. Aqueous organic solvents were found to give the highest values. Both, water and 100% acetone were the best solvents for obtaining extracts with higher TPC activities in MC fruit. However, with 50% Methanol the DPPH values were significantly ($P<0.05$) lower than both 70% and 100% acetone, and DPPH value where the three solvents showed significant differences ($P<0.05$).

For TPC and FRAP, the highest correlation (0.999) was observed in 100% acetone and water, while 100% methanol showed the lowest value. The TPC and DPPH in 70% methanol showed the highest correlation (0.992) while 50% ethanol showed the lowest value (0.589) and distill water showed the highest correlation (0.998). As for FRAP and DPPH the correlation in 70% methanol showed the highest correlation (0.997) while 50% ethanol showed the lowest value (0.686) and the distill water showed the highest correlation (0.906).

Table 1. Effect of different extraction solvents on the antioxidants activities from *M. charantia* fruit determined by DPPH radical-scavenging activity, and ferric-reducing antioxidant power (FRAP) and total phenolic contents

		DPPH	FRAP	TPC
Acetone	50%	34.48±10.02^{f}	13.01±0.36^{g}	11.31±0.28^{f}
	70%	83.10±0.29^{b}	32.18±0.31^{c}	20.08±0.41^{b}
	100%	79.56±0.24^{c}	33.61±0.45^{b}	21.35±0.39^{b}
Ethanol	50%	34.22±0.50^{f}	16.76±0.12^{f}	12.85±0.12^{f}
	70%	82.48±0.59^{b}	29.48±0.48^{d}	18.55±0.04^{d}
	100%	77.74±0.34^{d}	33.91±0.48^{b}	19.70±0.73^{c}
Methanol	50%	68.85±0.58^{e}	25.27±0.59^{e}	16.43±0.33^{e}
	70%	82.84±0.32^{b}	32.36±0.96^{c}	18.56±0.13^{d}
	100%	77.85±0.32^{d}	33.54±0.54^{b}	19.06±0.41^{c}
Water		98.29±2.02^{a}	38.92±2.05^{a}	23.30±0.10^{a}

Data represents mean±SD (n=3). Different lowercase letters in the same column indicate significant difference at $P \leq 0.05$

Table 2. Antioxidant potentials of *M. charantia* fruit assayed by FRAP and TPC assay in plasma and liver of experimental and normal male rat

		M. charantia	Control
Plasma	FRAP	3.9229±.0747	3.4729±.10989^{b}
	TPC	1.6800±.02035^{a}	1.5343±.02816^{b}
Liver	FRAP	1.7857±.07527^{a}	1.4614±.03744^{b}
	TPC	1.9071±.01017^{a}	1.7943±.00997^{b}

Data represents mean±SD (n=7). Different lowercase letters in the same column indicate significant difference at $P \leq 0.05$

The total antioxidant capacity of their plasma was measured by FRAP assay. Plasma antioxidant levels increased significantly after consumption of *M. charantia* fruit compared to the control group. There was significant change in liver homogenate antioxidant FRAP values over the same period after ingestion of *M. charantia* fruit compared to the control group (Table 2). However, total phenolic content of plasma and liver homogenate were significantly highest compared to the control group.

Discussion

Several methods have been used to determine antioxidant activity of plants. Our present study therefore involved three various established methods to evaluate antioxidative activity of *M. charantia*, namely, DPPH radical-scavenging activity, hydroxyl radical- scavenging activity, ferric reducing/ antioxidant power (FRAP) assay and total antioxidant capacity. Phenolic compounds were identified and total phenol content was also determined.

This study demonstrated that the antioxidant activity could be determined by using several test systems. However, there are several methodological limitations for antioxidant determination (Kaur and

Kapoor, 2001). To measure the antioxidant capacity of bitter gourd, we suggest that FRAP is an appropriate method. Methanol has stronger polarity than the chloroform solvent (Rahmat *et al*., 2003). In this study, however, the high antioxidant activity of *M. charantia* methanolic extract may be due to the majority of the active compounds in the MC fruit being dissolved in the methanolic solvent (strong polar) instead of the chloroform solvent (relatively-nonpolar). The DPPH assay is one of the most common and relatively quick methods used for testing radical scavenging activity of various plant extracts (Lillian *et al*., 2008).

The methanol extract in this study was significantly ($P<0.05$) lower than the water extract. This results agreed with that of Ansari *et al*. (2005) who reported that, water extract of MC showed higher free radical antioxidant activities than a cold extract of MC The scavenging ability of an extract is dependent on the active components contained in it. This therefore shows that methanol extract has more active constituents more than the ethanol extract. These results agree with the results of Nabila *et al*. (2008) with their experiments on the antioxidant and antimicrobial activities of the Pistacia lentiscus and Pistacia atlantica extracts.

Statistical correlations have been studied between total phenol content and antioxidant activity determined by different assays. Total phenol content was shown to provide the highest association with FRAP assay in the present study ($R^2 = 0.999$). This supports the results reported by Guo *et al*. (2003) for different fractions of fruits. This result was also in agreement with Benzie and Stezo (1999), who found a strong positive correlation between total phenolic content and FRAP assay.

The FRAP method was applied in this study, which appeared to be highly reproducible and suitable to detect small increases in antioxidant activity. Recently the same FRAP method was used in a study in which significant increases in plasma antioxidant capacity could be detected following the consumption of wine (Duthie *et al*., 1998). The results of the present study indicated that MC consumption caused a significant increase in both plasma FRAP and TPC ,the increase in plasma antioxidant capacity observed after consumption of flavonoid-rich foods often greatly exceeds the increase in plasma flavonoids(Silvina and Balz, 2004). Other studies have reported such effects, in particular an acute increase in the antioxidant capacity of plasma (Leenen *et al*., 2000; Pedersen *et al*., 2000; Mazza *et al*., 2002), we investigated the effects of MC consumption in male rats on plasma antioxidant levels and total antioxidant capacity. We discovered that very similar increases in plasma antioxidant capacity could be achieved by consumption of MC fruit.

In conclusion, the present study has demonstrated that *M. charantia*. rich in phenolics and have a strong antioxidant activity and a radical-scavenging action in all of the tested methods. And we concluded the increase in plasma total antioxidant capacity observed after the consumption of dietary *M. charantia* suggested that *M. charantia* is a good source of natural antioxidants. Malaysian bitter melon might have health benefits for consumers as a potential functional food or value-added ingredient.

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