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Antioxidant Activity and Cytotoxicity of Bitter Melon (*Momordica charantia* L.) Extract Cultured in Lampang Thailand

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ABSTRACT

The ethanol extract of bitter melon (*Momordica charantia* L.) grown in Lampang province, Thailand was investigated for its total phenolic content, antioxidant activity and cytotoxicity, respectively. The bitter melon extract (BME) was prepared from whole plant which consists of leaves, stems and root power. The amount of total phenolic content (TPC) was measured using the Folin-Ciocalteu reagent method. To evaluate antioxidant activity, 2,2-diphenyl-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays were performed. The results showed that BME extract has a high phenolic content about 49.42±1.28 mg gallic acid equivalents (GAE). In addition, BME possessed similar radical scavenging activity as compared to ascorbic acid by the DPPH method and Fe reducing activity by FRAP assay was 32.23±0.96 mg/g ascorbic acid equivalent. The cytotoxicity shown by Lactate dehydrogenase (LDH) assay of the BME on cervical cancer cell line was 0, 51 and 98% at concentrations of 80, 100 and 120 µg/ml for Hela cells and 0, 30 and 70% at concentrations of 140, 160 and 180 µg/ml for Siha cells. From all of the results, this BME showed strong antioxidant activity and moderate cytotoxicity on cervical cancer cells (Hela and Siha cells). Therefore, the non-toxic concentrations of BME will be used to test the effect of BME on invasion and progression of cervical cancer cell lines.

Keywords: Bitter melon, antioxidant activity, total phenolic compound, cervical cancer

Introduction

Momordica charantia L. (bitter melon, bitter gourd) has been commonly eaten as a vegetable in Thai food with Thai chili paste and also used as a traditional Thai herb and the fruits eaten as vegetable with Thai chilli paste. It is used as an alternative therapy to treat many symptoms such as stomach-intestine disorders, ulcers, colitis constipation and intestinal worms. It is also well known as a medicine for diabetes, antiviral, antineoplastic and antioxidative (Basch *et al*., 2003 and Waiyaput *et al*., 2012). It is an immunomodulator in HIV positive people (Chunthorng-Orn *et al*., 2012 and Fang *et al*., 2012).

Previous studies of Bitter Melon Extract (BME) on cervical cancer were incomprehensive. There were only 2 evidences showed that leaf extract from bitter melon can reverse multidrug resistance (MDR) phenotype in human epidermal

carcinoma cell line (KBV-1) (Limtrakul *et al*., 2004 and Pitchakarn *et al*., 2012) and another one said bitter melon ingestion combined with radiotherapy affected to level and function of natural killer (NK) cells in cervical cancer patients by enhancing the percentage of NK cells and reducing P-gp level on NK cell membrane (Pongnikorn *et al.*, 2003). For other related studies, it was shown that BME can inhibit human breast cancer cells (both cell lines and human primary cells) proliferation and induced apoptotic death (Ray *et al.*, 2010). Interestingly, no-to-low side effects of BME in animals as well as in humans were also published (Nerurkar and Ray, 2010).

This time, we study about the antioxidant activity of BME determined by DPPH and FRAP assays. The cytotoxicity test on Hela and Siha cells was also evaluated as a cell model related to cervical cancer.

Methodology

2.1 Chemicals and reagents

Hela cells (Adenocarcinoma, HPV 18 positive) was a gift from Assoc. Prof. Dr. Prachya Kongtaweelert, Faculty of Medicine, Chiangmai University. Siha cells (Squamous cell carcinoma, HPV 16 positive) were obtained from Assoc. Prof. Dr. Jamsai Peinthong, Faculty of Medicine, Khon Kaen University. Absolute ethanol and dimethyl sulfoxide (DMSO) were bought from Merck Ltd., Thailand. Folin-Ciocalteau's reagent, 2,2-diphenyl-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-S-triazine (TPTZ), acetate buffer, and ferric chloride ($FeCl_3$) were purchased from Sigma-Aldrich (Thailand) Co., Ltd. Minimum Essential Media (MEM), Penicillin Streptomycin and fetal bovin serum (FBS) were bought from Gibco by gibthai (Thailand). LDH Cytotoxicity Kit II was obtained from Promokine, Germany. Gallic acid, ascorbic acid, and other chemicals were of analytical grade.

2.2 Plant collection and extraction

The bitter melon leaves, stems and roots were baked and powdered at Lampang Herb Conservation Factory. The preparation was decontaminated to be free from bacteria and fungus. Preparation processes are guaranteed by Thai Food and Drug Administration (Thai FDA). A 100 g of bitter melon powder was extracted with 400 ml of 80% ethanol at 37 °C for 16 hrs. Extract was filtered with vacuum filter system. The filtrate was bleached with 16 g of active charcoal and dried under vacuum by rotary evaporation. A lyophilized form of the extract was resuspended in DMSO before used. Stock solutions were 2 mg/ml for anti-oxidant assays and 10 mg/ml for cytotoxicity test.

2.3 Total phenolic content assay

The amount of total phenolic content (TPC) in the BME was measured using the Folin-Ciocalteu reagent method (Singleton and Orthofer, 1999). The BME (0.2 ml, 0.01 mg/ml distilled water) was mixed with 1 ml of the Folin-Ciocalteau reagent (10% v/v) and incubated for 5 mins before adding 3 ml of 20% (w/v) sodium carbonate. After mixing the solution, the mixture was incubated at room temperature in a dark room for 2 hrs with intermittent shaking. The absorbance was measured at 765 nm using an ultraviolet-visible (UV/VIS) spectrophotometer (Jassco V530,

Japan). The samples were tested in triplicate. To gain a calibration curve, the standard sample used was five concentrations of diluted gallic acid solution. The amounts of TPC in BME were expressed as gallic acid equivalents in milligram per gram (mg GAE/g) of dry extract.

2.4 DPPH radical scavenging assay and IC_{50} determination

The radical scavenging activity of BME was determined using a DPPH (Brand-Williams *et al.,* 1995). Briefly, the distilled water (DW)-diluted solutions of the BME was prepared from a concentrated extract stock (2 mg/ml, dried extract in DMSO) into five concentrations to perform a concentration plot. Then, each concentration (2 ml) of BME was mixed with 2 ml DPPH in methanol (0.004%) for 30 min in a dark room temperature. The absorbance was measured at 517 nm using an ultraviolet-visible (UV/VIS) spectrophotometer (Jassco V530, Japan). The ascorbic acid was used as the positive standard. All BME samples and the standard were tested in triplicate. The scavenging activities of all samples were calculated as the percent inhibition of DPPH radical scavenged activity using the following formula:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The IC_{50} was calculated from fifty percent inhibition against concentration (µg/ml) plot.

2.5 Ferric reducing antioxidant power (FRAP) assay

Reducing power capacity was determined using FRAP assay (Benzie and Strain, 1996). Briefly, 0.1 ml of five different concentrations of BME (prepared from a stock solution described in DPPH section) was mixed with 3 ml of FRAP reagent (300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ), 20 mM $FeCl_3$ [10:1:1]. The mixture was incubated in a dark room for 20 mins and the absorbance was recorded at 593 nm using an ultraviolet-visible (UV/VIS) spectrophotometer (Jassco V530, Japan). In standard preparation, six concentrations of ascorbic acid were plotted to determine the reducing power capacity.

2.6 Cytotoxicity on Hela cells and Siha cells

Hela and Siha cells were grown and maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, sodium bicarbonate, 100 units/ml of Penicillin and 100 µg/ml of Streptomycin at 37°C with 5% CO_2 atmosphere in humidified incubator. Cells passages 53 to 65 were used in this study. 5×10^4 Cells were cultured in 96-well plates in each well for 24 hrs. The supernatant was removed and the extracts (different concentrations in media: 60-140 µg/ml for Hela cells and 120-200 µg/ml for Siha cells) were added to wells and continue cultured for 24 hrs. 10 µl of supernatant from each concentration of extracts was reacted with LDH- cytotoxicity test kit. The cytotoxicity to Hela, Siha monolayers is shown as relative amount being given by the summation of LDH releasing in the

supernatant compared to High control and Low control. Calculation of the percentage cytotoxicity using the formula of:

$$\text{Cytotoxicity (\%)} = \frac{\text{(Test Sample-Low Control)}}{\text{(High Control-Low Control)}} \times 100$$

Low control: cells grown in MEM with 2% of DMSO
High control: cells grown in MEM with 10% of Cell Lysis Solution (included in LDH Kit which can destroy cell membrane to let LDH release out)

Results and Discussion

3.1 Total phenolic content and antioxidant capacity of BME

As far as we searched in literatures, single part of fruits, leaves, or seeds of bitter melon has been extracted with either ethanol or water to screen its total phenolic content and antioxidant activity including cytotoxicity (Ma *et al*., 2014, Chunthorng-Orn *et al*., 2012, Patel *et al.*, 2011 and Kumar *et al*., 2010). This study demonstrated for the first time that the ethanol extract (a mixture of leaves, stems and roots) of bitter melon (BME) possessed both the total phenolic content and antioxidant capacity. Herein, the amount of total phenolic content of BME is 49.42 ± 1.28 mg gallic acid equivalent/g of dried BME extract as determined from the calibration curves of the gallic acid ($Y = 5.4081\ x + 0.093$, $R^2 = 0.9968$). As compared to the BME from the fruit (Patel *et al.*, 2011), BME in this study contains greater amount of total phenolics which was around 8 folds. In DPPH scavenging assay (Table 1), the BME possessed a concentration-response relationship in the DPPH scavenging activity which was similar to the trend of ascorbic acid. Recent result showed that the IC_{50} of BME was 508.91±17.19 µg/ml as compared to that of ascorbic acid. However this value is higher (around 5 folds) than that of ethanol fruit extract (Patel *et al.*, 2011). For FRAP capacity assay (Table 1), the displayed concentration-dependent increases in the reducing power. The result showed that the Fe reducing activity of BME was 23.23 ± 0.96 mg/g ascorbic acid equivalent as determined from the calibration curves of standard ascorbic acid ($Y = 0.007\ x + 0.3769$, $R^2 = 0.9802$). This reducing activity is comparable to the ethanol fruit extract demonstrated from previous studies (Chunthorng-Orn *et al*., 2012 and Kumar *et al*., 2010). These results showed R-squared (R^2) with greater than 95% indicating that our results were very hight accurate. We conclude that not only BME fruit but also it whole tree posses the total phenolic content and antioxidant capacity.

3.2 Cytotoxicity test

The cytotoxicity effect of the bitter melon extracted by ethanol on Hela and Siha cells was evaluated by LDH-cytotoxicity test kit. Cytotoxicity is usually increased by the quantification of cell membrane damage. LDH is an enzyme which is found in all cell types and quickly released in to cell culture medium when cell membrane damaged. The results showed that cytotoxicity on Hela cells increased with concentration at 60, 80, 100, 120 and 140 µg/ml produced effects of 0, 0, 51, 98 and 100% respectively (Figure 1). Thus, a range of non-toxic concentrations 10-80 µg/ml

will be used to treat Hela cells in the further experiment on effect of BME on cervical cancer progression *in vitro.* Also, the cytotoxicity on Siha cells also increased with concentration at 120, 140, 160, 180 and 200 µg/ml caused effects of 0, 0, 30, 70 and 71% (Figure 2). From this result, 20-140 µg/ml of BME will be selected for Siha cells treatment in further experiment too. Besides, the cytotoxicity of BME to Siha cells was tested by using concentrations up to 500 µg/ml and 100% of cytotoxicity was found at around 400-500 µg/ml (data not shown). We actually search for the non-toxic concentrations for the next study and the results show at non-toxic concentrations (cytotoxicity 0%) is significantly different from toxic concentrations (Figure 1 and 2). Siha cells originated from Squamous cell carcinoma (HPV 16 positive) whereas Hela cells originated from Adenocarcinoma (HPV 18 positive). In Thailand, Squamous cell carcinoma is more predominant (69%) than Adenocarcinoma (25%). Recently, adenocarcinoma incidence tends to increase due to HPV 16, 18 infections in young Thai women and higher taking estrogen hormone. Our next plan is to use non-toxic concentrations of BME to treat both cervical cancer cell lines to see the effect of BME on invasion and migration of cancer including to matrix metalloproteinase (MMP) expression. From cytotoxicity results, BME has less cytotoxicity to Siha cells than Hela cells. Therefore, Adenocarcinoma may be more sensitive to BME treatment than Squamous cell carcinoma.

Table 1 Antioxidant activities of bitter melon extracts as determined by IC_{50} of DPPH and FRAP assay

Antioxidant activity assays	Samples		Calibration curve, coefficient of Determination (R^2)
	BME	Ascorbic acid	
IC_{50} of DPPH (µg/ml)	508.91± 17.19	8.06 ± 0.22	Y = 0.0922x + 3.0788, R^2 = 0.9942
FRAP (mg/g)	23.23 ± 0.96	32.27 ± 0.95	Y = 0.007x + 0.3769, R^2 = 0.9802

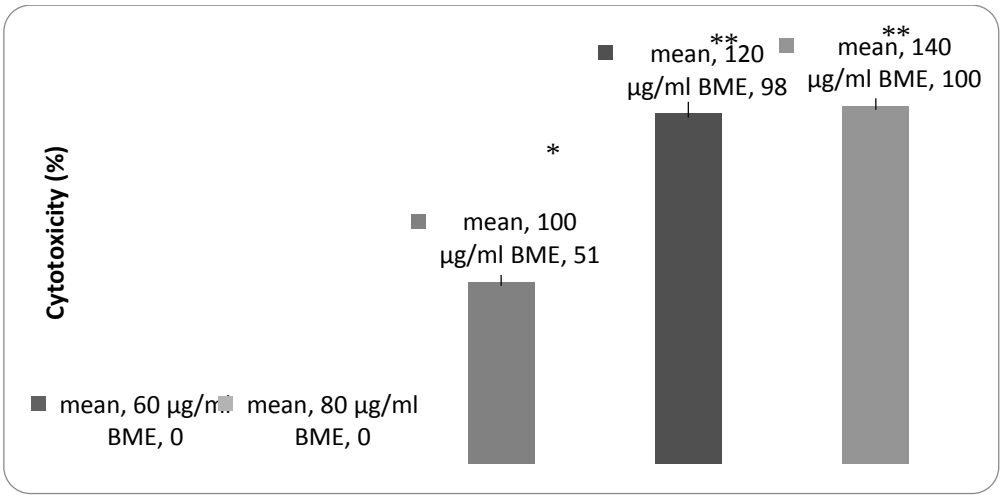

Figure 1 Cytotoxic effect of Bitter Melon extract on Hela cells. LDH amount released into media was measured after incubating Hela cells at 37°C for 24 hrs. % cytotoxicity of bitter melon extract was calculated compared to High Control (lysis buffer) subtracted to Low Control (DMSO).

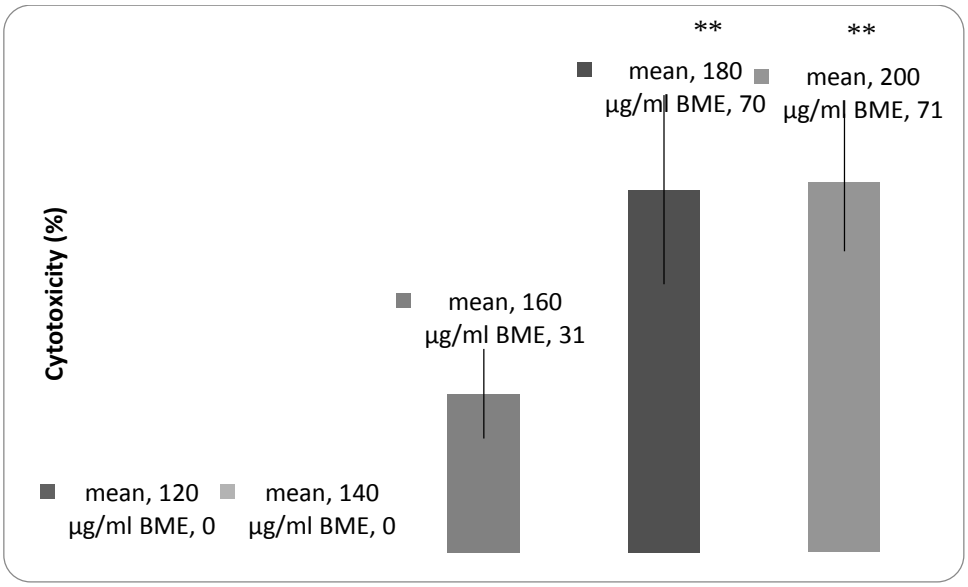

Figure 2 Cytotoxic effect of Bitter Melon extract on Siha cells. LDH amount released into media was measured after incubating Siha cells at 37°C for 24 hrs. % cytotoxicity of bitter melon extract was calculated compared to High Control (lysis buffer) subtracted to Low Control (DMSO).

Conclusion

In this study, the ethanol extract from a mixture of leaves, stems and roots of bitter melon has both a total phenolic content and an antioxidant capacity. The total phenolic content shown here was a 49.42±1.28 mg gallic acid equivalent. The antioxidant capacity was tested by DPPH scavenging assay and FRAP assay. The DPPH scavenging activity showed as IC_{50} of BME which was 508.91±17.19 µg/ml as compared to ascorbic acid (8.06±0.22 µg/ml). Moreover, the FRAP assay result showed Fe reducing activity of BME was 23.23±0.96 mg/g ascorbic acid equivalent as determined from the calibration curves of standard ascorbic acid. For cytotoxicity test, the non-toxic concentrations of BME will be used in the further experiments.

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