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Antioxidant and Cytoprotective Activities of Methanolic Extract from *Garcinia mangostana* Hulls

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ABSTRACT: *Garcinia mangostana* has been documented in Thai traditional medicine in various aspects. Although many benefits have been claimed, few scientific reports are available in the literature. In the present study, the methanolic extract from *G. mangostana* hulls (GME) was assessed for antioxidant and cytoprotective activities. The results showed that GME contained phenolic compounds and possessed reducing power as well as Fe^{2+} chelating activity. The antioxidant properties were determined by scavenging DPPH, nitric oxide and lipid radicals in dose-dependent manners. In particular, the powerful scavenging activities were found against hydroxyl and superoxide radicals when investigated using ESR spectrometry. GME also enhanced the cell survival by decreasing the oxidative damage in ECV304 endothelial cells after H_2O_2 exposure. These data indicated that GME played a pivotal role on the antioxidant and chemopreventive activities via a reducing mechanism and inhibition of intracellular oxidative stress, respectively.

KEYWORDS: Antioxidant activity, cytoprotective activity, electron spin resonance (ESR) spectrometry, *Garcinia mangostana*.

INTRODUCTION

Free radicals in aerobic organisms play a crucial role in the development of tissue damage and pathological events. In healthy organisms, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are approximately balanced by both enzymatic and non-enzymatic antioxidant defense mechanisms[1]. If this process is inadequate for scavenging reactive species completely, it will lead to potential oxidative damage to important macromolecules of cells. It is generally accepted that various degenerative disorders are caused by oxidative damage such as cardiovascular diseases, aging, Parkinson's disease, Alzheimer's disease, Huntington's disease, atherosclerosis, stroke and cancer[2].

The concept of disease-chemoprevention has been regarded as one of the most processing avenues for disease control. In recent years, considerable effort has been directed towards identifying naturally occurring substances that can protect against oxidative stress. There has been a worldwide trend towards the use of natural phytochemicals present in fruits, vegetables, oil seeds, teas, herbs, berry crops and beans. Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals and alteration of intracellular redox potential[3].

Garcinia mangostana Linn. (mangosteen), family Guttiferae, is an indigenous plant in Thailand and some other Southeast Asian countries. It is one of the most popular fruits named 'the queen of fruits'. It has been known to be of good medicinal value and is traditionally used in folk medicines for treatment of abdominal pain, diarrhea, dysentery, infected wounds, suppuration, chronic ulcer, leucorrhoea and gonorrhoea[4]. Phytochemical studies showed that hulls of this plant contained various secondary metabolites, such as tannins, triterpenes, anthocyanins, xanthones, polysaccharides, phenolic compounds, vitamins B1, B2 and C, and other bioactive substances[5]. Moreover, recent scientific studies reported that *G. mangostana* possessed several biological and pharmacological properties such as antihistamine, anti-fungal, anti-bacterial, anti-HIV-1 protease and induction of apoptosis in cancer cell lines[6]. Although several properties of *G. mangostana* have been studied, few authentic scientific reports are available, when compared with other medicinal plants.

The aims of this study were to investigate the antioxidant properties of methanolic extract from *G. mangostana* hulls (GME) towards $DPPH^{\bullet}$, nitric oxide ($^{\bullet}NO$) and lipid ($L^{\bullet}$) radicals by chemical methods in test tubes. In addition, hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) scavenging activities were determined using electron spin resonance (ESR) spectrometry. Reducing ability,

total phenolic contents and an active constituent were quantitated. Interestingly, this is the first report to discover the cytoprotective activity of GME on human ECV304 endothelial cells using the MTT assay to study the survival rate of H_2O_2 treated cells and DCFH-DA fluorescent probe to study the inhibition of intracellular oxidative stress. We hope that our data will be the basis to propose the preliminary mechanisms of radical scavenging activities related to the cytoprotective activity of *G. mangostana* extract.

Materials and Methods

Plant material and extract preparation

Garcinia mangostana hulls were collected from Chantaburi Province, Thailand, during the summer time in May 2004. Fruits were cleaned with running tap water and fresh hulls were separated and chopped into pieces. They were dried under shade at room temperature for 5 days and the air-dried hulls were then ground to powder for extraction.

An amount of 1 kg of powdered sample was extracted with 5 L of methanol for a week with maceration at 37°C. The extract was then collected and filtered through Whatman No.1 filter paper in a Buchner funnel under vacuum. The filtrate was concentrated by evaporation with a vacuum rotary evaporator at 45°C to yield 183.8 g of dried methanolic extract. The dried extract was stored at 4°C for further uses. The methanolic extract was standardized by quantifying the amount of α-mangostin, an active compound, using HPLC equipped with the BDS Hypersil C18 column (46 mm x 250 mm). The extract solution (5 ml) was injected in each run with 0.1% ortho-phosphoric acid and acetonitrile as mobile phase at 1 ml/min, 25°C. The HPLC system was precalibrated with α-mangostin standard (5-500 μg/ml) to evaluate the linear correlation curve. The amount of α-mangostin was determined from the calibration curve of α-mangostin and the peak area of each concentration of the samples and calculated by the following linear equation:

$$\text{Area} = (11006 \times [\alpha\text{-mangostin}]) - 42616, R^2 = 0.9998$$

Total phenolic contents

The extract was diluted with the same solvent used for extraction, to a suitable concentration for analysis. Total phenolic contents of GME were assessed approximately by using the Folin-Ciocalteu phenol reagent method[7]. The extract (1 ml) was added with 10% Folin-Ciocalteu reagent and 0.5% sodium carbonate (0.5 ml). The contents were thoroughly mixed and allowed to stand for 30 min. Absorption at 750 nm was measured in a spectrophotometer. The total phenolic contents were expressed as gallic acid equivalents in milligram per gram of the extract, using a standard curve generated with gallic acid. The concentrations of gallic acid (GA; mg/ml) in the reactions were analyzed from the following calibration curve equation, determined by linear regression:

$$\text{Absorbance} = (21.51 \times [GA]) + 0.1004, R^2 = 0.9608$$

Reducing power

The Fe^{3+} reducing power of the extract was determined according to the method of Oyaizu[8]. The extract (2 ml) was mixed with 0.2 M phosphate buffer, pH 6.6 (2 ml) and 1% potassium ferricyanide (2 ml). The mixture was then incubated at 50°C for 20 min. Afterwards, the mixture was stopped by adding 10% trichloroacetic acid (2 ml) and then centrifuged at 3,000 rpm for 10 min. The upper layer of supernatant (2 ml) was mixed with distilled water (2 ml) and 0.1% $FeCl_3$ solution (0.5 ml). The absorbance was measured at 700 nm against a blank with a spectrophotometer, and ascorbic acid was used as a standard. Higher absorbance of the reaction mixture indicated greater reducing power. The percents of reducing power were presented as ascorbic acid equivalents using a calibration curve between the absorbance of the reaction and the percent of the reducing power ability of ascorbic acid:

$$OD = (0.0146 \times [\text{percent}]) + 0.0016, R^2 = 0.9999$$

Ferrous ion chelating activity

The chelating of ferrous ion by the extract was estimated by the method of Gülçin[9]. Briefly, the extract (2 ml) was added to a solution of 2 mM $FeCl_2$ (0.1 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 5 min. Absorbance was measured at 562 nm and EDTA was used as a positive control. The percents of inhibition of ferrozine-Fe^{2+} complex formation were given by the following formula:

$$\text{Ferrous ion chelating activity}\,(\%) = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 was the absorbance of the control (the mixture without the extract), A_1 was the absorbance of the mixture in the presence of the extract and A_2 was the absorbance without ferrozine.

DPPH scavenging activity

The antioxidant activity of the extract was estimated on the basis of the radical scavenging effect of the stable DPPH•[10]. Various concentrations of the extract were added to a methanolic 0.4 mM DPPH• solution (0.1 ml) in a 96 well plate. The reaction mixture was shaken vigorously and allowed to stand for 30 min at 37°C. The degree of DPPH• purple decolorization to DPPHH yellow indicated the scavenging efficiency of the extract. The absorbance of the mixture was

determined at 517 nm using UV-Vis microplate reader and ascorbic acid was served as a positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The scavenging activity against DPPH• was calculated using the following equation:

Scavenging activity (%) = $[1 - (A_1 - A_2) / A_0] \times 100\%$

where A_0 was the absorbance of control (DPPH• solution without the extract), A_1 was the absorbance of DPPH• solution in the presence of the extract and A_2 was the absorbance without DPPH• solution.

Hydroxyl radical scavenging activity

The •OH was measured by spin trapping •OH with DMPO and the resultant DMPO-OH adduct was detected with an ESR spectrometry[11]. The ESR spectra were measured at room temperature after mixing 0.02 ml of 0.1 mM H_2O_2 with 0.01 ml of 2 M DMPO, 0.05 ml of the extract and 0.02 ml of 0.05 mM Fe^{2+} using the X-band ESR spectrometer (JES-RE1X, JEOL, Kyoto, Japan). ESR spectrometer setting parameters were as follows: external magnetic field 337.5 mT ± 5 mT, field modulation 0.1 mT of 100 kHz, microwave 10 mW of 9.43 GHz. Ascorbic acid and ethanol were used as controls. The hydroxyl radical scavenging rate of the extracts was calculated by the following equation:

Scavenging rate = $[(h_0 - h_x) / h_0] \times 100\%$

where h_x and h_0 were the ESR signal intensities of reactions in the presence and absence of the extract, respectively.

Superoxide radical scavenging activity

In the ESR spin trapping, the generated superoxide radicals were trapped by DMPO to form the spin adducts, DMPO-OOH using hypoxanthine-xanthine oxidase system and detected with X-band ESR spectrometry[11]. The mixture contained 0.01 ml of 4 mM hypoxanthine, 0.01 ml of 20 mM DTPA, 0.01 ml of 2 M DMPO, 0.05 ml of the extract and 0.02 ml of 0.4 U/ml xanthine oxidase. ESR spectra were measured at room temperature with ESR spectrometry under the above conditions. Superoxide dismutase (SOD) was used as a control. The superoxide radical scavenging rate of the extract was calculated by the following equation:

Scavenging rate = $[(h_0 - h_x) / h_0] \times 100\%$

where h_x and h_0 were the ESR signal intensities of reactions in the presence and absence of the extract, respectively.

Nitric oxide radical scavenging assay

Sodium nitroprusside (SNP) in aqueous solution at physiological pH, spontaneously generated •NO which interacted with oxygen to produce nitrite ion, which was estimated using Griess reagent[12]. The reaction mixture containing 2 ml of the extract at different concentrations and 50 mM SNP (0.5 ml) in 10 mM PBS was incubated at 37°C for 60 min. An aliquot (0.5 ml) of the incubation solution was pipetted out and diluted with 0.5 ml of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (NED). The absorbance of the chromophore that formed during diazotization of nitrite with sulfanilamide and subsequent coupling with NED was immediately recorded at 540 nm. The absorbance from various concentrations of sodium nitrite salt treated the same way with Griess reagent was plotted for a standard curve. The amount of NO_2^- (μM) was calculated from the following calibration curve equation, determined by linear regression:

Absorbance = $(0.0276 \times [NO_2^-]) + 0.1162$, $R^2 = 0.9823$

The capability to scavenge •NO radicals was calculated using the following equation:

Scavenging activity (%) = $[1 - (A_1 - A_2) / A_0] \times 100\%$

where A_0 was the absorbance of the control (the reaction mixture without the extract), A_1 was the absorbance in the presence of the extract and A_2 was the absorbance without Griess reagent. α-Tocopherol was used as a standard.

Antioxidant activity in linoleic acid emulsion system

The degree of oxidation due to the formation of alkoxy radicals by the redox reaction with ferrous ions (reducing agents) in a linoleic acid emulsion at physiological pH was measured by the thiocyanate method[13]. The reaction mixture containing 0.5 ml of the extract, 0.2 M sodium phosphate buffer, pH 7.0, (2 ml) and linoleic emulsion (2.5 ml) was incubated at 37°C. Aliquots (1 ml) were taken at different intervals during incubation which was further diluted with 75% ethanol (4.7 ml), 200 mM $FeCl_2$ (0.1 ml) in 3.5% HCl and 30% KSCN. The resulting red chromogen of the ferric (III)-thiocyanate complex could be measured at 500 nm. The inhibition of lipid peroxidation (LPI) in percent was calculated by the following equation:

LPI (%) = $[1 - (A_1 - A_2) / A_0] \times 100\%$

where A_0 was the absorbance of control (the reaction mixture without the extract), A_1 was the absorbance in the presence of the extract, and A_2 was the absorbance without potassium thiocyanate solution. α-Tocopherol was used as a standard.

Cell culture

Human umbilical vein endothelial cell line ECV304, obtained from the American Type Culture Collection (Rockville, MD), was grown in RPMI 1640 medium supplemented with fetal bovine serum, 100 units/ml of penicillin G and 0.1 mg/ml of streptomycin sulfate in a humidified atmosphere of a 5% CO_2 at 37°C.

Cytoprotective activity

Cell viability was determined by measuring the metabolism of a tetrazolium substrate MTT[6]. Cells were seeded on 96-well plates at a density of $2x10^4$ cells/well and incubated for 24 h. The medium was then aspirated and replaced with various concentrations of the extract for 1 h. Afterwards, medium was removed and incubated with 0.1 ml of H_2O_2 (1 mM) for 6 h. After exposure to H_2O_2, the MTT solution (1 mg/ml) was incubated with cells for 60 min. MTT in solution is converted to a blue formazan crystal by mitochondrial succinate dehydrogenase of living cells. The crystal was solubilized from cells with isopropanol and its absorbance determined at 590 nm. The percents of cell viability were calculated with the following equation:

Cell viability (%) = (A_1 / A_0) x 100%

where A_0 was the absorbance of the negative control (cells without the extract) and A_1 was the absorbance in the presence of the extract.

Inhibition of intracellular reactive oxygen species (ROS)

Intracellular ROS production was estimated using fluorescent DCFH-DA probe[14]. Briefly, 2 x 10^5 cells/ml were pre-treated with various concentrations of the extract for 1 h prior to DCFH-DA (0.01 mM) exposure for 30 min. H_2O_2 (1 mM, 500 ml) was then added and incubated at 37°C for 1 h. Intracellular ROS production was evaluated by spectrofluorometer (Perkin-Elmer, MA). The percent of ROS production was calculated according to the following equation:

Relative amount of intracellular ROS (%) = (FI_1 / FI_0) x 100%

where FI_0 was the fluorescence intensity of the negative control (cells without the extract) and FI_1 was the fluorescence intensity in the presence of the extract at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Statistical analysis

All the experiments were carried out in triplicate and the results were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and the Student *t* - test. The values of $P < 0.01$ and $P < 0.05$ were considered statistically as significant. The amount of the effective concentration of the extract needed to inhibit free radicals by 50%, IC_{50}, was graphically estimated by interpolation from linear regression analysis between the scavenging activities (%) versus various concentrations of the extract.

Results

Total phenolic contents

It was well-known that plant phenolics are highly effective free radical scavengers and antioxidants. *G. mangostana* contained high amounts of xanthones, a class of phenolic compounds (Fig 1). In this study, the contents of total phenolics in GME were determined spectrometrically according to the Folin-Ciocalteu method and calculated as gallic acid equivalents (GAE). It was shown that GME contained phenolic compounds at 60.2 mg GAE. This result indicated that the potent antioxidant activity of GME may be related to the phenolic compounds in the extract.

Reducing power

The direct correlation between antioxidant activity and reducing power of certain plant extracts were reported. The presence of reductants (antioxidants) in the extracts would result in the reduction of iron (III) to iron (II). The amount of iron (II)-ferricyanide complex could be determined by measuring the formation of Perl's Prussian blue at 700 nm[15]. It was found that the reducing ability of GME against Fe^{3+} increased with increasing concentration. GME showed 50% of activity

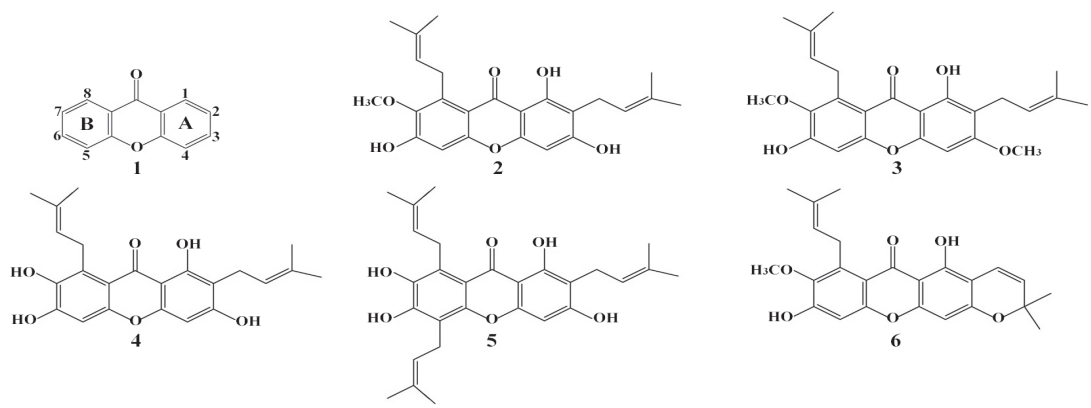

Fig 1. The important xanthones of *G. mangostana*: (1) nucleus of xanthone; (2) α-mangostin; (3) β-mangostin; (4) γ-mangostin; (5) garcinone E; (6) 9-hydroxycalabaxanthone.

(IC_{50}) at 84.35 μg/ml, while ascorbic acid had an IC_{50} of 65.7 μg/ml. The result indicated that GME contained electron donors and possessed the ability to reduce iron (III) to iron (II) in a concentration-dependent fashion (Table 1).

Ferrous ion chelating activity

Ferrozine could quantitatively form complexes with Fe^{2+}. In the presence of chelating agent, the complex formation was disrupted with the result that the red color of the complex was decreased. Measurement of color reduction, therefore, allowed the estimation of the chelating activity of the coexisting chelator. As shown in Table 1, GME interfered the formation of Fe^{2+}- ferrozine complex and its activity showed an IC_{50} value at 19.26 μg/ml. This indicated that GME captured Fe^{2+} before it formed the complex with ferrozine and the activity of GME was 3-times less than EDTA (IC_{50} value 6.49 μg/ml).

DPPH scavenging activity

The DPPH assay was used to preliminarily screen for antioxidant activity of the extract. The proton-radical scavenging action is known as an important mechanism of antioxidants. GME decolorized the purple of DPPH$^{\bullet}$ to the yellow of DPPHH in a dose-dependent manner with an IC_{50} value of 20.50 μg/ml (Table 1). Although the DPPH$^{\bullet}$ radical-scavenging ability of GME was below that of ascorbic acid (IC_{50} value = 13.57 μg/ml), it was evident that GME did show the high proton-donating ability on DPPH$^{\bullet}$ to form stable DPPHH molecules.

Hydroxyl radical scavenging activity

Hydroxyl radical ($^{\bullet}OH$) was produced by the Fenton reaction and trapped by DMPO. The typical 1:2:2:1 4-line ESR spectra of the DMPO-OH adduct were observed as shown in Fig 2. The correlation curve of scavenging ratio (%) and the concentrations of the extract was plotted to estimate its ability. When the amount of $^{\bullet}OH$ radicals was diminished, the peak high of spectrum was reduced. GME scavenged $^{\bullet}OH$ in a dose-dependent manner with an IC_{50} value of 47 μg/ml. In the other hand, GME showed strong activity to inhibit the generation of $^{\bullet}OH$ radicals from the Fenton reaction.

Superoxide radical scavenging activity

In Fig 3, $O_2^{\bullet -}$ scavenging activity was investigated with the hypoxanthine-xanthine oxidase system and trapped by DMPO. The typical ESR signals of the DMPO-OOH spin adduct were detected. The correlation curve of the peak height and the concentration was calculated

Table 1. Antioxidant activities of methanolic extract from *Garcinia mangostana* (GME).

Method	Sample	Concentration(μg/ml)	Activity (%)	IC_{50}(μg/ml)
RP	GME	5	4.30 ± 1.28	84.35
		25	19.40 ± 1.06	
		100	56.35 ± 0.93	
		200	86.75 ± 2.11	
	Asc	100	77.42 ± 2.08	65.71
FC	GME	5	40.51 ± 0.27	19.26
		25	54.43 ± 0.01	
		100	78.12 ± 3.37	
		200	84.86 ± 2.90	
	EDTA	10	73.98 ± 4.20	6.49
DPPH	GME	5	19.76 ± 3.05	20.50
		25	64.36 ± 0.20	
		100	84.38 ± 4.12	
		200	84.13 ± 2.10	
	Asc	100	85.43 ± 2.50	16.00
NO	GME	5	10.68 ± 2.14	55.61
		25	34.49 ± 0.55	
		100	61.39 ± 1.64	
		200	67.95 ± 0.47	
	Toc	100	62.05 ± 1.60	24.98
LPO	GME	5	22.75 ± 1.51	9.43
		25	61.97 ± 1.80	
		100	71.74 ± 1.57	
		200	75.44 ± 1.67	
	Toc	100	75.74 ± 2.04	34.01

RP, reducing power presented as ascorbic acid equivalents; FC, ferrous ion chelating activity;
DPPH, DPPH scavenging activity; NO, nitric oxide scavenging activity; LPO, Inhibition of lipid peroxidation;
Asc, Ascorbic acid; Toc, α-Tocopherol; GME, Methanolic extract from *G. mangostana* hulls; IC_{50},
the concentration at which 50% of the maximum activity was produced.

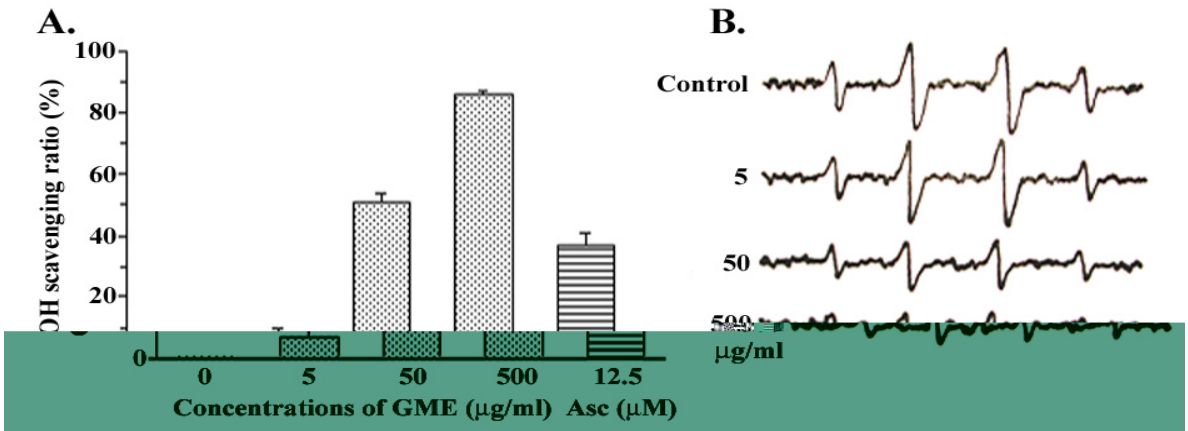

Fig 2. The scavenging effect of GME on hydroxyl radicals was determined with the Fenton reaction and measured using ESR spectroscopy. (A) The OH$^{\bullet}$ scavenging activity of GME was shown in a dose-dependent manner and compared with ascorbic acid (Asc). (B) ESR spectrum of spin adduct of DMPO-OH was demonstrated to study the effect of GME on OH$^{\bullet}$.

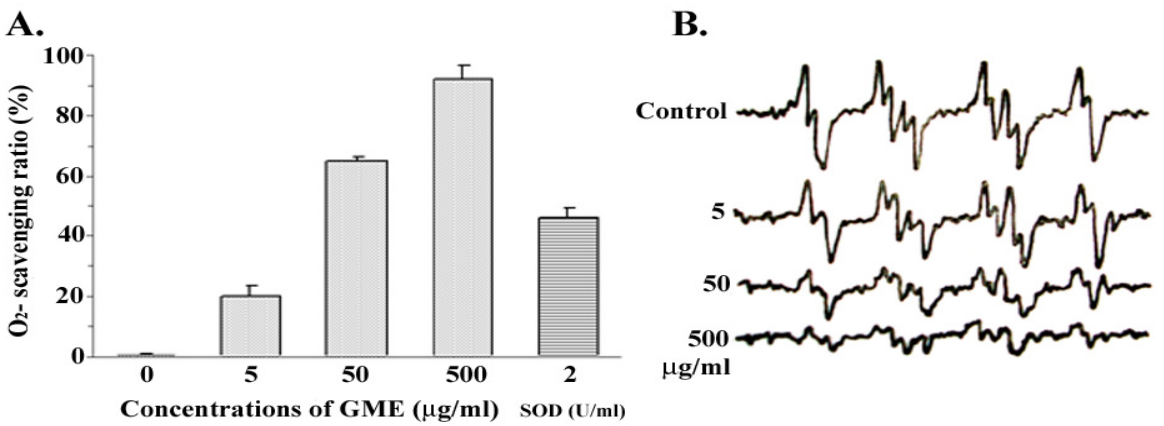

Fig 3. The scavenging effect of GME on superoxide radicals was determined with hypoxanthine-xanthine oxidase system and measured using ESR spectroscopy. (A) The $O_2^{\bullet-}$ scavenging activity of GME was shown in a dose-dependent manner and compared with superoxide dismutase (SOD). (B) The ESR spectrum of the spin adduct of DMPO-OOH was determined to study the effect of GME on $O_2^{\bullet-}$.

to find the ability of the extract. GME possessed powerful $O_2^{\bullet-}$ scavenging activity in a dose-dependent manner with an IC_{50} value of 25 μg/ml. We found that GME acted as $O_2^{\bullet-}$ scavenger better than $^{\bullet}$OH scavenger.

Nitric oxide scavenging activity

Formation of nitric oxide by SNP in aqueous solution at physiological condition has been reported to occur by spontaneous oxidation. $^{\bullet}$NO is a very unstable species, under aerobic condition it reacts with oxygen to produce, through intermediates such as NO_2, N_2O_4, N_3O_4, the stable products NO_3^- and NO_2^-. $^{\bullet}$NO radicals were determined indirectly with Griess reagent through the inhibition of nitrite production. As shown in Table 1, GME possessed $^{\bullet}$NO scavenging activity with an IC_{50} value of 55.61 μg/ml, while α-tocopherol had an IC_{50} value of 24.98 μg/ml.

Inhibition on lipid peroxidation

GME not only exhibited excellent ability for various radical scavenging activities, but also suppressed lipid peroxidation. GME successfully inhibited the oxidation of linoleic acid which was determined by a ferric thiocyanate (FTC) assay. The result demonstrated that GME inhibited lipid peroxidation in a concentration-dependent manner with an IC_{50} value of 9.43 μg/ml, while α-tocopherol showed IC_{50} value at 34.01 μg/ml (Table 1).

Cytoprotective activity

The cytoprotective activity of GME was studied on ECV304 cells after exposure with H_2O_2 as a cytotoxic agent and oxidative stress inducer. As shown in Fig 4, H_2O_2 decreased cell viability and its cytotoxic effect was attenuated in the presence of GME. The result

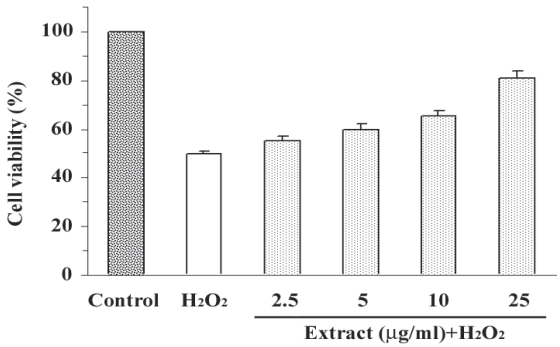

Fig 4. Cytoprotective effect of GME on H_2O_2-induced ECV304 cells was determined by the MTT assay. Cell controls without extract and H_2O_2 exposure had 100% of cell viability (▩), whereas cells treated with H_2O_2 but without extract showed the lowest percent of cell viability (☐). The pre-incubation of the extract on cells prior to H_2O_2 (1 mM) treatment was determined to be the cytoprotective (▨). The data were expressed as mean ± SD of triplicate measurements. A *P* value < 0.05 demonstrated significant cytoprotective activity.

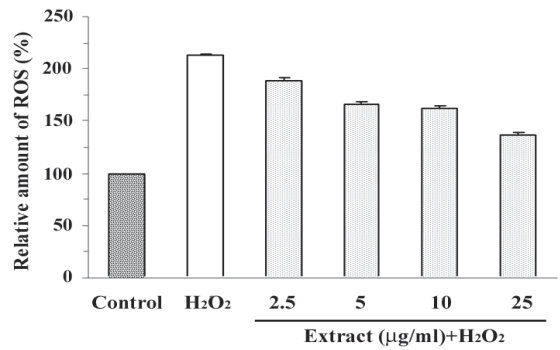

Fig 5. Effect of GME on ROS production in ECV304 cells as determined using DCFH-DA fluorescent probe. Cell controls were cultured without extract and H_2O_2 exposure (▩), whereas the highest oxidative stress-induced cells were grown in the presence of H_2O_2 (1 mM) without extract (☐). The inhibition of ROS production in cells was conducted by pre-incubating the extract with cells prior to H_2O_2 treatment and detected by the DCFH-DA probe (▨). The relative amount of ROS (%) is shown as mean ± SD of triplicate measurements. A *P* value < 0.05 displayed significant inhibitory activity.

demonstrated that GME at 2.5-25 μg/ml exhibited significant cytoprotective effects on human ECV304 endothelial cells. The cell viability increased to 80.9% at 25 μg/ml GME ($P < 0.05$), while the viability of H_2O_2-treated cells without GME was 49.7%.

Inhibition on intracellular oxidative stress

Accumulation of intracellular ROS was detected with DCFH-DA, which is freely permeable to cell membranes. Once inside cells, DCFH-DA is hydrolyzed by the esterase activity to DCF, which can be trapped intracellularly. DCF was then able to interact with peroxides and form the fluorescent 2′,7′-dichlorofluorescein, which was readily detected by spectrofluorometer[14]. H_2O_2 was used to produce the oxidative stress in human endothelial ECV304 cells. As shown in Fig 5, the intracellular ROS accumulation resulting from H_2O_2 exposure was significantly reduced in the presence of GME at 2.5-25 μg/ml ($P < 0.05$) in a dose-dependent manner.

Discussion

Phenolic compounds, such as benzoic acid derivatives, are commonly found in the plant kingdom and have been extensively exploited because of their multiple biological activities, including antioxidant effects. Since they contain at least one hydroxy-substituted aromatic ring system, they can form chelate complexes with metal ions and are easily oxidized, as well as serving as important units for donating electrons[16]. In the current study, it was found that GME contained phenolic compounds at 60.2 mg GAE. It was proposed here that the phenolic compounds of GME may play an important role in the observed antioxidant activities through free radical quenching, electron transfer, radical addition or radical recombination in various tested systems. The majority of phenolic compounds in the fruit hull of *G. mangostana* belong to the xanthone family, which may help to offset chronic diseases related with ROS. Two major xanthones were found in the fruit hull of this plant namely α-mangostin and γ-mangostin[17]. In this study, α-mangostin, one of the major and interesting active compounds from *G. mangostana* was identified and quantitated with HPLC chromatography to standardize the quantity and quality of the extract. α-Mangostin was present in our GME at 25.19 ± 0.22 g/100 g of GME. Recently, the biological activities of the α-mangostin and γ-mangostin xanthones were revealed. α-Mangostin showed antiproliferative activity against human leukemia HL60 cells through apoptotic cell death[18], while γ-mangostin inhibited the activity of cyclooxygenases (both COX-1 and COX-2) and suppressed the conversion of arachidonic acid to prostaglandin E2[19]. However, there has been no previous report on the antioxidant activities of xanthones from this plant against various kinds of radical species to reveal the important mechanism. From this study, we assumed that xanthones in the extract may have an important role on antioxidant activity.

To verify the radical scavenging ability of GME, several methods were served to investigate its ability

compared with commercial antioxidants against radical species by UV-Vis and ESR spectroscopy. Starting from the study on reducing ability, the result showed that GME expressed reducing ability as an electron donor and possessed the ability to reduce iron (III) to iron (II) in a concentration-dependent fashion. The reducing power of GME might be due to the di- and monohydroxyl substitutions in the aromatic ring of phenolic compounds, which possessed potent hydrogen donating abilities, and breaking of the free radical chain through donating a hydrogen atom[20].

Several antioxidants possess metal chelating activity to reduce the redox potential and stabilize the oxidized form of the metal ions, which related to the obstruction on the peroxidative process and oxidative damage. However, chelating activity might not be the main role of the extract, because its activity was 3-times less than EDTA which was used as a standard (IC_{50} value = 6.49 μg/ml). This result indicated that compounds in GME were not potent iron chelators; it could largely not obstruct the generation of $^{\bullet}OH$ radicals from the Fenton reaction. $^{\bullet}OH$ radicals were still produced from the reaction between iron (II) and H_2O_2. The more important role of GME might be related to the scavenging of $^{\bullet}OH$ after it was generated from the Fenton reaction, whereby it would inhibit the reaction of the radicals against biomolecules.

DPPH$^{\bullet}$ radicals have been used extensively as stable radicals to preliminarily evaluate the antioxidant activities of plant extracts. GME did show the potent proton-donating ability on DPPH$^{\bullet}$ to produce DPPHH, which is an important mechanism of antioxidants. Xanthones in the extract could transfer labile H-atoms to radicals. Lee et al[21] investigated the antioxidant activity of the xanthones of *Cudrania tricuspidata*. It was found that a xanthones of *C. tricuspidata* (cudraxanthone D), which contain a dihydroxyl group in the B-ring, exhibited strong radical scavenging activities. Such a structure is found in γ-mangostin and garcinone E of *G. mangostana*. On the other hand, cudraxanthone C of *C. tricuspidata* showed lower activity because one of its hydroxyl groups in the vicinal diol was protected, as found in α-mangostin, β-mangostin and 9-hydroxycalabaxanthone of *G. mangostana*. It could be rationalized that O-protected xanthones could not transfer to quinone, while those with vicinal dihydroxyl groups could transfer to quinone easily by releasing two electrons in the molecule. On the other hand, such a xanthone could easily donate electrons or protons to radical molecules to scavenge and quench the toxicity of the radicals.

In this study, the radical scavenging activity of GME against $^{\bullet}OH$ and $O_2^{\bullet-}$ was determined by spin trapping technique and measuring with ESR spectrometry. $^{\bullet}OH$ radicals were generated by the Fenton system ($Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$). Therefore, $^{\bullet}OH$ scavenging activity could be influenced by electron-donating and metal-chelating abilities of xanthones in GME. $^{\bullet}OH$ is widely implicated as the most potent oxidant and the major damaging species in free radical pathology. It can initiate lipid peroxidation, cause DNA strand breaks, and indiscriminatingly oxidize virtually any organic molecule[22]. This result revealed that GME possessed powerful $^{\bullet}OH$ scavenging activity in Fig 2, while Fe^{2+} chelating activity was 3-times less than EDTA. It could be explained that the active compounds in GME might have the main role to scavenge $^{\bullet}OH$ radicals generated from Fenton reaction by donating electrons. Although α-mangostin and β-mangostin as O-protected xanthones might show low DPPH scavenging activity, these xanthones might unexpectedly possess powerful $^{\bullet}OH$ radical scavenging activity due to their chelating effect with Fe^{2+} which prevents the generation of $^{\bullet}OH$ radicals in the system.

$O_2^{\bullet-}$ is the first reactive oxygen radical produced by one-electron reduction of molecular oxygen in metabolism process and the source of other radicals. $O_2^{\bullet-}$ scavenging activity was investigated with the hypoxanthine-xanthine oxidase system and trapping with DMPO[10]. The typical ESR signals of the DMPO-OOH spin adduct were detected, as shown in Fig 3. Experimental data demonstrated that GME exhibited much stronger scavenging activity against $O_2^{\bullet-}$ radicals compared with $^{\bullet}OH$ radicals. It was likely that GME might well show an inhibition of oxidative damage in the early stage to protect and/or balance the physiological system by concerting H-atom abstraction (electron donation) and catalyzing the dismutation of $O_2^{\bullet-}$ (protonation effect).

Abnormally high level of $^{\bullet}NO$ has been implicated in various inflammatory and degenerative diseases. Inflammatory cells, which were components of atherosclerotic plaques, produced extracellular $^{\bullet}NO$ which presumably interfered with the endothelial proliferation[23]. Thus, the pathophysiology of the endothelium was modulated by $^{\bullet}NO$. Our indirect method to investigate $^{\bullet}NO$ scavenging activity was based on the release of $^{\bullet}NO$ from SNP in physiological solution and determined with Griess reagent through the inhibition of nitrite production. The decreased level of nitrite in the reaction indicated an increased scavenging effect against $^{\bullet}NO$ since the amounts of nitrite were directly proportional to the amounts of $^{\bullet}NO$ radicals in the reaction mixture. GME activity was weaker than the standard α-tocopherol in the system. However, the result indicated that some compounds in GME scavenged $^{\bullet}NO$ in the reaction, which might influence the protection of cells, especially endothelial cells, by neutralizing the $^{\bullet}NO$ produced in oxidative stress.

Oxidation of unsaturated fatty acid in biological

membranes leads to the formation and propagation of lipid radicals (L•), the uptake of oxygen, the rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids to produce breakdown products such as malondialdehyde, which is known to be mutagenic and carcinogenic[24]. Lipid peroxidation is a key process in many pathological events and is one of the reactions induced by oxidative stress. The GME prevented lipid peroxidation, which may explain its cytoprotective property on cell membrane damage caused by radicals or toxic substances.

To verify the cytoprotective activity of GME, the MTT assay was used to determine viability after exposure of cells to H_2O_2 as a cytotoxic agent and oxidative stress inducer. GME pretreatment was able to protect cells and increase the number of surviving cells significantly. Interestingly, accumulation of intracellular ROS using DCFH-DA fluorescent dye was decreased in GME-pretreated cells after exposure to H_2O_2. This could be explained as GME inhibition of intracellular oxidative stress and protection of the ECV304 cells from oxidative damage. Thus, GME-pretreated cells survived after exposure with H_2O_2.

In conclusion, the results obtained from the present study clearly revealed that GME possessed antioxidant and cytoprotective activities through its abilities to scavenge various radicals produced in oxidative stress. Interestingly, the uniqueness of GME was related to cytoprotective activity in the initial step of lipid peroxidation in the cell membranes of ECV304 endothelial cells by the inhibition of superoxide radical generation and the donation of hydrogen atoms or electrons to other radical molecules such as hydroxyl radicals, nitric oxide radicals and lipid radicals. From the present study, it was found that GME contained xanthones as the main phenolic compounds which may possess antioxidant and cytoprotective activities. *G. mangostana* extract is a good starting source for further investigation on the exact constituents responsible for these activities. Our preliminary study on isolation of methanolic extract compounds, including the chemical and biological identification, is now in process. We hope that the intensive study on the outcoming active constituents of *G. mangostana* will lead to the discovery of a novel botanical-drug for chemoprevention.

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