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Inhibitory effect of aqueous extracts from Miracle Fruit leaves on mutation and oxidative damage

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ABSTRACT

This study investigated the inhibitory effects of aqueous extracts from Miracle Fruit leaves (AML) on mutation and oxidative damage. The results showed that AML in the range of 1–5 mg/plate inhibited the mutagenicity of 2-aminoanthracene (2-AA), an indirect mutagen, and 4-nitroquinoline-*N*-oxide (4-NQO), a direct mutagen toward *Salmonella typhimurium* TA 98 and TA 100. On the other hand, AML in the range of 0.05–0.2 mg/ml showed radical scavenging, reducing activities, liposome protection as well as decreased *tert*-butyl hydroperoxide (*t*-BHP) induced oxidative cytotoxicity in HepG2 cells. High performance liquid chromatography (HPLC) analysis suggested that the active phenolic constituents in AML are *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans*-*p*-coumaric acid and veratric acid. These active phenolic components may contribute to the biological protection effects of AML in different models. The data suggest that AML exhibiting biological activities can be applied to antimutation as well as anti-oxidative damage.

1. Introduction

The production of DNA damage plays an important role on mutation and ageing diseases. Various mutagens present on food increase oxidative stress and cancer risk through different mechanism in cells. For example, 4-nitroquinoline-*N*-oxide (4-NQO), a direct and strong mutagen, is a quinoline derivative carcinogen and can also induce potent intracellular production of oxidative stress (Arima et al., 2006). In addition, 2-aminoanthracene (2-AA), a known carcinogenic polycyclic aromatic amine, can induce tumours primarily in the liver (Baker et al., 2001). In Ames test, 2-AA requires metabolic activation by the S9 liver preparation. The metabolic products of these mutagens chiefly bind to DNA at guanine residues. The consequence is that whenever these DNA adducts is generated, DNA mutation may increase, eventually increasing the risk of tumour progression.

Except DNA damage, these mutagens may be metabolized and induce harmful oxidative stress, which also destroy the biological molecules (e.g. lipids) and cause mutations (Takabe et al., 2001). However, many reports suggest that intracellular oxidative stress derived from reactive nitrogen species (RNS) and reactive oxygen species (ROS) arises during physiological metabolism and after exposure to various chemical encouragements. For example, *tert*-butyl hydroperoxide (*t*-BHP), an organic hydroperoxide, can be converted into free radicals by cytochrome P450 enzymes (Minotti, Borrello, Palombini, & Galeotti, 1986). Therefore, *t*-BHP is often used as a model compound for inducing oxidative stress during *in vitro* and *in vivo* studies. Further, oxidative stress can be observed in different pathological states, such as atherosclerosis and cancer. Therefore, the inhibition of oxidative stress may play an important step in preventing mutation and ageing diseases. On the other hand, to decrease the oxidation of lipids, various antioxidants have been used to protect the lipids in foods from oxidation. However, because of safety concerns, there is currently an interest in replacing synthetic antioxidants with natural antioxidants. Thus, investigations on the natural inhibitors of oxidation in foods have received much attention.

The Miracle Fruit shrub, *Synsepalum dulificum* Daniell, bears red berries which contain a taste-modifying protein, miraculin (Theerasilp & Kurihara, 1988). Previous studies suggested that Miracle Fruit contain high amounts of bioactive compounds, such as phenolic acids (Wang, Chou, et al., 2011; Inglett & Chen, 2011). Earlier studies suggested that Miracle Fruit berries can improve insulin resistance induced by fructose-rich chow in rat (Chen, Liu, & Cheng, 2006). To our knowledge, our study is the first to examine

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the biological effects of Miracle Fruit leaves, an agricultural waste material, on mutation and oxidation. The objective of this work is to determine the antimutagenic and anti-oxidative activities of aqueous extract from Miracle Fruit leaves.

2. Materials and methods

2.1. Materials

4-Nitroquinoline-*N*-oxide (4-NQO), 2-aminoanthracene (2-AA), thiobarbituric acid (TBA) and *tert*-butyl hydroperoxide (*t*-BHP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA) and chloromethylfluorescein-diacetate (CMF-DA) were purchased from Invitrogen Molecular Probes (Grand Island, NY, USA). Culture medium and top agar was prepared as previously described (Wang, Duh, Wu, & Huang, 2011). The Miracle Fruit leaves were harvested from commercial plantations in Kaohsiung, Taiwan during the July of 2012.

2.2. Sample preparation

The Miracle Fruit leaves were grounded after freeze-drying. The powder (10 g) was extracted with water (200 ml) at 100 °C for 30 min and then centrifuged at 10,000*g* for 20 min. The extract was filtered and the residue was re-extracted under the same conditions. The combined filtrate was then freeze-dried. The yield obtained was 8.86% (w/w). The final sample was named as AML (the aqueous extract of Miracle Fruit leaves).

2.3. DPPH radical inhibition assay

The effect of samples on the DPPH radical was estimated as described previously (Hatano, Kagawa, Yasuhara, & Okuda, 1988). The samples (0.1–0.4 mg/ml, 1 ml) were added to a methanolic solution (1 ml) of DPPH radical (final concentration of DPPH was 0.2 mmol/l). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min; the absorbance of the resulting solution was then measured at 517 nm.

2.4. Reducing activity assay

The reducing power of AML was determined as previously described (Oyaizu, 1986). The sample (0.1–0.4 mg/ml, 2.5 ml) were added to potassium ferricyanide (2.5 ml, 10 mg/ml), and the mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 ml, 100 mg/ml) was added to the mixture, which was then centrifuged at 650*g* for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 1.0 mg/ml), and then the absorbance was read at 700 nm. The reducing activity was calculated against an ascorbic acid calibration curve.

2.5. Liposome oxidation assay

A solution containing the lecithin (500 mg) and phosphate buffer (50 ml, 10 mM, pH 7.4) was sonicated by an ultrasonic cleaner (Branson 8210, Branson Ultrasonic Corporation, Danbury, CT, USA) in an ice-cold water bath for 2 h. The sonicated solution (1 ml), $FeCl_3$ (0.12 mM, 1 ml), ascorbic acid (0.5 mM, 1 ml) and AML (0.2–0.8 mg/ml, 1 ml) were mixed and incubated at 37 °C for 1 h. The levels of liposome oxidation were determined as previously described (Wang, Duh, et al., 2011).

2.6. Measurement of HepG2 cells viability

HepG2 cells (ATCC No. CRL-11997) were purchased from Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in minimum essential medium (MEM) containing 10% fetal bovine serum and maintained in humidified 5% CO_2/95% air at 37 °C. After cells were cultured with AML (final concentration was 0.05–0.2 mg/ml), in the presence of 0.2 mM *t*-BHP or not for 6 h, cell viability was determined by the 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Wang, Lee, Chen, Yu, & Duh, 2012).

2.7. Evaluation of reactive oxygen species (ROS) and glutathione (GSH) in HepG2 cells

DCF-DA was used to determine the generation of ROS in HepG2 cells. The cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) is a chemically reduced form of fluorescein used as an intracellular indicator for reactive oxygen species (ROS) production in cells. Upon cleavage of the acetate groups by intracellular esterases the nonfluorescent DCF-DA is converted to DCFH that need an oxidation via H_2O_2 and a peroxidase to be transform in the highly fluorescent 2′,7′-dichlorofluorescein (DCF). HepG2 cells were pretreated with DCF-DA (50 μM) for 30 min, and then AML (final concentration was 0.05–0.2 mg/ml) were added to the medium in the presence of 0.2 mM *t*-BHP or not. After incubation at 37 °C for 3 h, the adherent cells were trypsinized and washed with normal saline. ROS produced from intracellular stress was detected using a Bio-Tek FLx800 microplate fluorescence reader (Winooski, VT, USA) with excitation and emission wavelengths of 485 and 530 nm, respectively (Wang et al., 2012). On the other hand, intracellular GSH levels were determined after staining cell with chloromethylfluorescein-diacetate (CMF-DA) (Wang et al., 2012). CMF-DA form GSH adducts in a reaction catalysed by glutathione-S-transferase. After conjugation with GSH, CMF-DA is hydrolysed to the fluorescent 5-chloromethylfluorescein by cellular esterase. HepG2 cells were treated with AML (final concentration was 0.05–0.2 mg/ml) in the presence of 0.2 mM *t*-BHP or not. After incubation at 37 °C for 3 h, the adherent cells were trypsinized and washed with normal saline. Intracellular GSH was detected using a Bio-Tek FLx800 microplate fluorescence reader (Winooski, VT, USA) with excitation and emission wavelengths of 485 and 530 nm, respectively (Wang et al., 2012).

2.8. Mutagenicity assay

The mutagenicity of AML was tested according to the Ames test with a 20 min first incubation at 37 °C (Maron & Ames, 1983). The histidine-requiring strains of *Salmonella typhimurium* TA 98 and TA 100 were obtained from Taiwan Agricultural Chemicals and Toxic Substances Research Institute (Taichung, Taiwan). The external metabolic activation system, S9 mix (Molecular Toxicology, Inc., Boone, NC, USA) was prepared from Sprague–Dawley male rats treated with Aroclor 1254. Samples (0.1 ml, 10–50 mg/ml corresponding to 1–5 mg/plate) were added to the overnight cultured *S. typhimurium* TA 98 or TA 100 (0.1 ml) and S9 mix (0.5 ml) or 0.1 M phosphate buffer (0.5 ml, pH 7.4) in place of the S9 mix. The entire mixture was incubated at 37 °C for 20 min before molten top agar (2.0 ml) was added and then spread out in a Petri dish containing 20 ml of minimum agar. The mixture was counted after incubating at 37 °C for 48 h. The toxic effects of AML on *S. typhimurium* TA 98 and TA 100 was determined as previously described (Wang, Duh, et al., 2011).

2.9. Antimutagenic activity assay

The antimutagenic activity of AML was assayed according to the Ames method except for the addition of mutagen before incubation (Maron & Ames, 1983). The concentrations of mutagens were tested as in a previous study (Mazzei et al., 2007). The mutagens used were 4-NQO (0.5 μg/plate), a direct mutagen and 2-AA (2.5 μg/plate), which required S9 mix for metabolic activation. Mutagen (0.1 ml) was added to the mixture of a strain (TA 98 or TA 100), and samples were added with the S9 mix for 2-AA or with phosphate buffer (0.1 M, pH 7.4) for 4-NQO. The mutagenicity of each mutagen in the absence of samples is defined as 100%. The number of spontaneous revertants in the absence of mutagens and samples was used as reference. The inhibition (%) of mutagenicity of the sample was calculated as following:

Inhibition (%) = {1 − [(No. of his^+ revertants with mutagen and sample − No. of spontaneous revertant)/(No. of his^+ revertants with mutagen − No. of spontaneous revertant)]} × 100.

2.10. High performance liquid chromatography (HPLC) assay

HPLC was performed with a Hitachi Liquid Chromatograph (Hitachi Ltd., Tokyo, Japan), consisting of two model L-7100 pumps, and one model L-7455 photodiode array detector. Sample (10 mg/ml) was filtered through a 0.45 μm filter and injected into the HPLC column. The injection volume was 20 μl and the flow rate was 0.8 ml/min. The separation temperature was 25 °C. The column was a Mightysil RP-18 GP (5 μm, 250 × 4.6 mm I.D.; Kanto Corporation, Portland, OR, USA). The method involved the use of a binary gradient with mobile phases was as previously described (Wang, Duh, et al., 2011). The plot of the peak-area (y) vs. concentration (x, μg/ml), the regression equations of the three marker compounds and their correlation coefficients (r) were as follows: *p*-hydroxybenzoic acid, $y = 0.1812x + 0.1414$ ($r^2 = 0.9994$); vanillic acid, $y = 0.1037x + 0.0983$ ($r^2 = 0.9992$); syringic acid, $y = 0.0477x + 0.03$ ($r^2 = 0.9995$); *trans*-*p*-coumaric acid, $y = 0.032x + 0.0091$ ($r^2 = 0.9991$); and veratric acid, $y = 0.0939x + 0.0665$ ($r^2 = 0.9991$).

2.11. Statistical analysis

All data were presented as means ± standard deviations (SD). Each test was performed in triplicate. Statistical analysis involved use of the Statistical Analysis System software package (SAS Institute Inc.). Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $p < 0.05$.

3. Results

The HPLC chromatographic analysis showed that bioactive phenolic components were presented in AML. These phenolic components have been identified as *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans*-*p*-coumaric acid, and veratric acid by measuring their retention time and UV absorbance, in relation to standards (Fig. 1). Thus, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans*-*p*-coumaric acid, and veratric acid were selected as marker compounds for the HPLC fingerprint chromatograph of AML. The maximum absorbance of *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans*-*p*-coumaric acid, and veratric acid were 254.9, 291.0, 275.7, 307.2, and 260.5 nm. The relative amounts of these compounds found in AML were in the order syringic acid (2.91 mg/g of AML) > *trans*-*p*-coumaric acid (2.76 mg/g of AML) > vanillic acid (1.42 mg/g of AML) > *p*-hydroxybenzoic acid (1.39 mg/g of AML) > veratric acid (1.24 mg/g of AML). On the other hand, in Fig. 1, other major peaks from unknown compounds **A**, and **B** with retention time at 34.40, and 37.65 min, were observed,

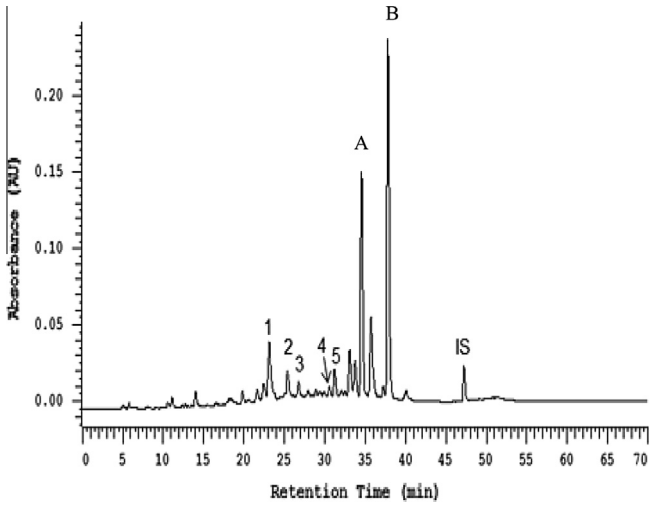

Fig. 1. HPLC chromatograms of the aqueous extract of Miracle Fruit leaves (AML). The peaks indicate the following 1, *p*-hydroxybenzoic acid; 2, vanillic acid; 3, syringic acid; 4, *trans*-*p*-coumaric acid; 5, veratric acid; IS, butyl p-hydroxybenzoate.

respectively. By ultraviolet–visible spectra analysis, compounds **A**, and **B** showed the maximum absorbance at 260.5 and 254.9 nm, respectively.

The effects of AML and the five marker components on radical scavenging and reducing activities are shown in Table 1. The reducing activity of natural products is regarded as their hydrogen donating capacity. The reducing ability of AML and its five marker components were determined in comparison with ascorbic acid. In the range 0.05–0.2 mg/ml, AML exhibited a reducing effect that increased, as the sample concentration increased. The reducing capacity of AML at 0.2 mg/ml was equivalent to 44.1 μg/ml of ascorbic acid. Among the five marker components, syringic acid and vanillic acid exhibited better reducing activities than *trans*-*p*-coumaric acid, *p*-hydroxybenzoic acid and veratric acid suppress did. The scavenging of DPPH radicals is a popular method for determining the antioxidant capacities of natural products. From 0.05 to 0.2 mg/ml, the DPPH radicals scavenging activity of AML was 55.1–99.3%, indicating that AML was a free radical scavenger. Table 1 also shows the inhibitory effects of *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans*-*p*-coumaric acid, and veratric acid at 0.01 mg/ml on DPPH radicals being 1.4%, 7.4%, 93.1%, 5.6% and 1.7%, respectively. These data indicated that the five marker constituents of AML and mainly syringic acid might play a part in the antioxidant activity as well as the reducing activity of AML.

Lipid oxidation can increase cellular damage and produce toxic metabolites. In this study, liposome protection was used as a measure of decreasing lipid oxidation provided by AML. As shown in Table 1, AML in concentrations of 0.05–0.2 mg/ml exhibited a 74.6–88.2% inhibitory effect on liposome oxidation induced by the Fe^{3+}/H_2O_2 system. Meanwhile, the inhibitory effects of *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans*-*p*-coumaric acid, and veratric acid at 0.01 mg/ml on lipid oxidation were 4.4%, 26.5%, 44.4%, 15.8% and 3.9%, respectively. These data indicated that AML and the five marker components, mainly syringic acid, provided effective protection from lipid oxidation in vitro.

To realise the cellular action of AML, the protective effects of AML on *t*-BHP-induced oxidative damage in HepG2 cells were detected. *t*-BHP, as a chemical toxin, could produce ROS intermediates in cells and promote lipid peroxidation, resulting in cellular injury. None of the samples in the range from 0.05 to 1 mg/ml showed any cytotoxic effect on the viability of HepG2 cells. On

Table 1
Effects of the aqueous extract of Miracle Fruit leaves (AML) on radical scavenging, reducing activity and liposome protection.

Sample	(mg/ml)	DPPH inhibition (%)	Reducing activity (μg Vit C/ml)	Liposome protection (%)
AML	0.05	55.1 ± 1.6	14.8 ± 1.8	74.6 ± 1.6
	0.1	90.8 ± 0.9	25.5 ± 0.6	80.3 ± 1.3
	0.2	99.3 ± 0.3	44.1 ± 2.9	88.2 ± 0.2
p-Hydroxybenzoic acid	0.01	1.4 ± 0.1	7.5 ± 0.4	4.4 ± 1.0
Vanillic acid	0.01	7.4 ± 0.7	16.5 ± 1.3	26.5 ± 0.7
Syringic acid	0.01	93.1 ± 1.1	28.7 ± 4.2	44.4 ± 1.1
trans-p-Coumaric acid	0.01	5.6 ± 1.2	7.8 ± 0.6	15.8 ± 1.7
Veratric acid	0.01	1.7 ± 0.1	6.4 ± 0.5	3.9 ± 0.7

Data represent means ± SD ($n = 3$).

the other hand, AML at the concentration of 2 mg/ml exhibited potential toxicity and decreased cell viability down to 59.6% of the control group (data not shown). As shown in Fig. 2, *t*-BHP at 0.2 mM decreased HepG2 cell viability to 36.7% of the control group. In the range of 0.05–0.2 mg/ml, AML protected liver HepG2 cells against oxidative damage in a dose-dependent manner. At the concentration of 0.2 mg/ml, AML increased cell viability up to 80.4% of the control group in the presence of *t*-BHP. Clearly, AML could protect the liver HepG2 cells against *t*-BHP induced oxidative cytotoxicity.

The levels of intracellular ROS in HepG2 cells induced by *t*-BHP were determined using a fluorescent probe. According to Fig. 3A, *t*-BHP significantly increased ROS generation compared to the control group. The intracellular ROS generation in HepG2 cells treated with *t*-BHP alone (100%) was 7.95-folds compared to the control group. The HepG2 cells treated with AML (0.05–0.2 mg/ml) in the presence of *t*-BHP decreased the intracellular ROS level to 71.3%, 56.5% and 36.5%, respectively, of the group treated with *t*-BHP alone. The ROS generation in HepG2 cells treated with AML decreased in a dose-dependent manner. However, AML displayed a potent suppression of oxidative cytotoxicity.

On the other hand, intracellular GSH depletion plays an important role in promoting cell death induced by oxidative damage. According to Fig. 3B, *t*-BHP decreased intracellular GSH level down to 59% of the control group (100%). Treatment of AML (0.05–0.2 mg/ml) in the presence of *t*-BHP increased intracellular GSH level to 70%, 89% and 91%, respectively, of the control group. As expected, AML could protect liver HepG2 cells against intracellular GSH depletion caused by *t*-BHP. This result implied that AML could inhibit intracellular GSH depletion in a cellular model and prevent ROS-induced oxidation.

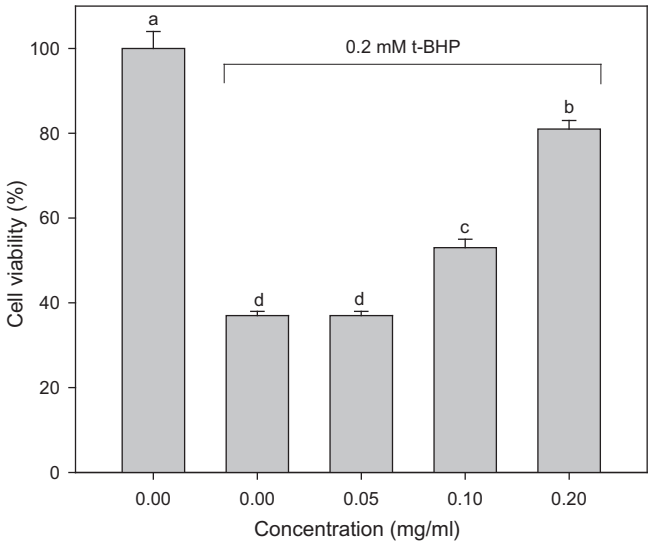

Fig. 2. Effects of the aqueous extract of Miracle Fruit leaves (AML) on *t*-BHP induced cytotoxicity in HepG 2 cells. Data are presented by means ± SD ($n = 3$). Values with different superscripts are significantly different ($p < 0.05$).

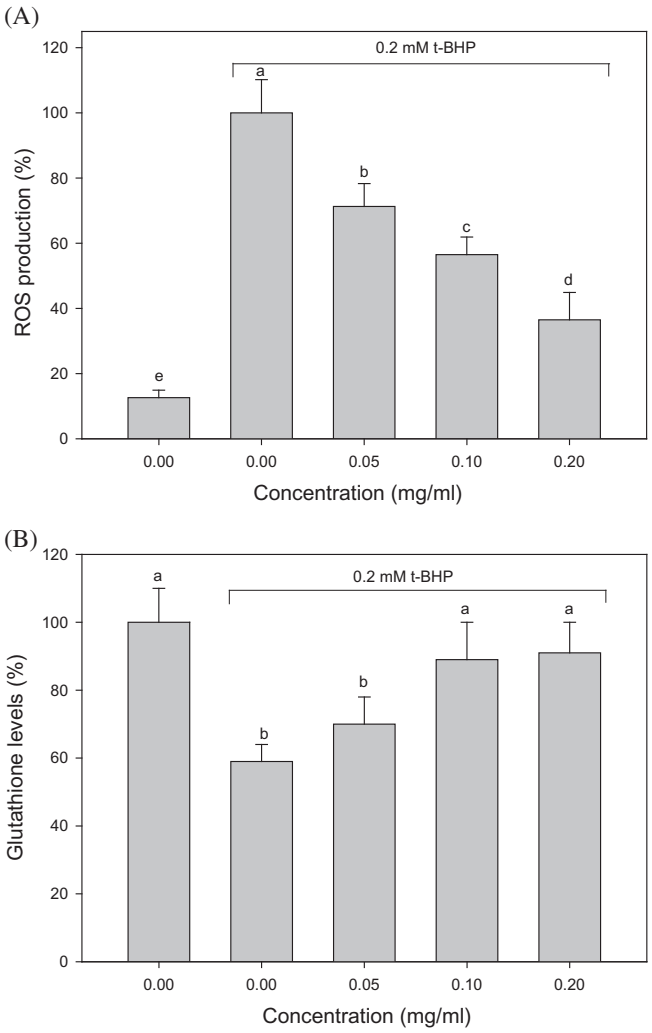

Fig. 3. Effects of the aqueous extract of Miracle Fruit leaves (AML) on *t*-BHP induced reactive oxygen species (ROS) (A) and glutathione (GSH) levels (B) in HepG2 cells. Data are presented by means ± SD ($n = 3$). Values with different superscripts are significantly different ($p < 0.05$).

The Ames assay is a common method for determining the mutagenicity of natural products. In the mutation study, if a lethal toxicity occurs in a test treated sample, the results of the mutagenicity could be compromised and the numbers of revertants of TA 98 and TA 100 would be inaccurate. In this study, AML (1–5 mg/plate) did not show any toxicity against TA 98 or TA 100 (data not shown).

The mutagenicity of AML was determined by comparing the ratio of induced revertants to spontaneous revertants in the plates. Table 2 shows that AML (1–5 mg/plate) did not significantly ($p > 0.05$) increase the number of colonies in *S. typhimurium* TA 98 and TA 100, with or without S9 activation. These observations indicated that AML did not increase the mutagenicity of *S. typhimurium* TA 98 and TA 100.

Furthermore, the antimutagenicity of AML on 4-NQO and 2-AA induced mutation in *S. typhimurium* TA 98 and TA 100 was examined. As shown in Table 3, AML displayed dose-dependent protection against 4-NQO induced mutagenicity in *S. typhimurium* TA 98 and TA 100, without S9 activation. AML at levels of 1–5 mg/plate showed 0–17% inhibition of 4-NQO induced mutagenicity in TA 98 and 2–58% inhibition in TA 100. Table 3 also shows the antimutagenicity of AML on 2-AA induced mutation in *S. typhimurium* TA 98 and TA 100, with S9 activation. AML at levels of 1–5 mg/plate showed 6–80% inhibition of 2-AA induced mutagenicity in TA 98 and 34–96% inhibition in TA 100. These observations indicated that AML could inhibit the mutagenicity of both direct and indirect mutagens *in vitro*.

Table 3
The antimutagenicity of the aqueous extract of Miracle Fruit leaves (AML) toward *S. typhimurium* TA 98 and TA 100.

Sample (mg/plate)	His^+ revertants/plate (% of inhibition)	
	TA 98 + 4-NQO	TA 100 + 4-NQO
0	679 ± 27 (0)^b	1895 ± 42 (0)^c
1	678 ± 18 (0)^b	1868 ± 38 (2)^c
2	660 ± 32 (2)^b	1439 ± 29 (27)^b
5	575 ± 22 (17)^a	901 ± 19 (58)^a
	His^+ revertants/plate (% of inhibition)	
	TA 98 + 2-AA + S9	TA 100 + 2-AA + S9
0	923 ± 32 (0)^d	1757 ± 53 (0)^d
1	873 ± 16 (6)^c	1232 ± 61 (34)^c
2	764 ± 24 (18)^b	769 ± 39 (64)^b
5	216 ± 18 (80)^a	266 ± 15 (96)^a

Data represent means ± SD ($n = 3$). 4-NQO, 4-nitroquinoline *N*-oxide. 2-AA, 2-anthramine. Values with different superscripts in a column are significantly different ($p < 0.05$). % of inhibition = {1 − [(No. of revertants with mutagen and sample − No. of spontaneous revertants)/(No. of revertants with mutagen − No. of spontaneous revertants)]} × 100. The number of spontaneous revertants was determined without samples and mutagen.

4. Discussion

In this study, AML demonstrated multiple biological activities, including antimutation and antioxidation. A considerable number of studies suggested that the effects of natural antioxidants, such as phenolic components of plant extract, in the biological systems provided protection because they scavenged radicals, chelated metals, and inhibited the oxidases and then regulate cellular redox states (Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010). Twelve phenolics were identified and quantified in the miracle berry flesh (Du, Shen, Zhang, Prinyawiwatkul, & Xu, 2014). Therefore, phenolic constitutes of AML such as *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans-p*-coumaric acid, and veratric acid were examined in this study.

As shown in Table 1, AML and its five marker components exhibited antioxidant activity by scavenging radicals. The phenolic constitutes of plant extracts could exhibit reactive species scavenger and regular oxidative stress. According to previous reports, sugarcane leaves have been considered to be an excellent dietary source of natural phenolic antioxidants (Wang, Duh, et al., 2011). It was suggested that any antioxidant capacity was due to the development of a reducing ability, when reacting with free radicals, which terminated the radical chain reaction (Huang, Ou, & Prior, 2005). As shown in Table 1, AML exhibited a reducing ability. This may be attributed to reducing compounds present in AML, which consequently react with free radicals, to stabilize, terminate radical chain reactions and decrease oxidation. For instance, syringic acid and vanillic acid showed greater reducing and radical scavenging effects than the other marker constituents did. Lipid oxidation occurs in cell membranes; it releases arachidonic acid, which is responsible for long-term oxidative stress in cells. In this study, liposome was prepared from phospholipid and used as a lipid oxidation model to imitate the lipid oxidation of biomolecules. AML demonstrated a protective effect against the lipid damage caused by the hydroxyl radicals produced from a Fenton-like reaction. In fact, 4-hydroxyl-2-nonenal (HNE), a harmful lipid oxidation product, can bind covalently to cellular DNA, to form the exocyclin etheno-DNA-base adduct (Nair, Bartsch, & Nair, 2007). In this study, the AML and its five marker components provided protection against lipid oxidation, indicating that AML could protect bio-lipid molecules from oxidative stress and prevent DNA damage in tissues. The equivalent concentrations of *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *trans-p*-coumaric acid, and veratric acid in 0.2 mg/ml of AML were 0.0003, 0.0003, 0.0006, 0.0006 and 0.0002 mg/ml, respectively. As shown in Table 1, it was possible that the synergistic effects might exist among the five marker components of AML in liposome protection. Previous studies conducted in order to evaluate the antioxidant activities of natural benzoic acid derivatives suggested a structure–activity relationship in model systems. The antioxidant potential depends on the substitution of the phenol ring with hydroxyl groups in ortho or para position. The methylation of the phenol ring in ortho position relative to the hydroxyl group also increased the activity. Therefore, compared to the other molecules detected by HPLC, syringic acid and vanillic acid have a more important effect on DPPH inhibition, reducing activity and liposome protection.

Oxidative stress would be generated during the metabolism of mutagens and carcinogens and could react with cellular molecules, and thereby promoting cancer progression. The HepG2 cell line is considered a good tool to study the toxic/cytoprotective effects of compounds to liver cells. *t*-BHP, as a well-known strong oxidant, is often used as a reference compound for inducing biological oxidative injury during different studies. The toxicity of *t*-BHP is related to its induction of mass peroxide production and reduction of intracellular glutathione levels (Wang et al., 2012). Therefore, *t*-BHP can be converted into ROS such as peroxyl and alkoxyl radicals by cytochrome P450 enzymes. It seems that the radical scavenging potential of AML contributed to its protective effects. Peroxidation of cell membrane lipids can decrease membrane fluidity and permeability and lead to the disruption of membrane structure and

Table 2
The mutagenicity of the aqueous extract of Miracle Fruit leaves (AML) toward *S. typhimurium* TA 98 and TA 100 with and without S9 mix.

Sample (mg/plate)	His^+ revertants/plate (% of spontaneous)	
	TA 98	TA 100
Spontaneous group	66 ± 9 (100)	175 ± 3 (100)
1	48 ± 3 (73)	166 ± 9 (95)
2	43 ± 4 (65)	173 ± 10 (99)
5	62 ± 3 (94)	187 ± 5 (107)
	TA 98 + S9	TA 100 + S9
Spontaneous group	69 ± 3 (100)	203 ± 31 (100)
1	62 ± 6 (90)	198 ± 9 (98)
2	65 ± 4 (94)	208 ± 3 (102)
5	74 ± 2 (107)	216 ± 28 (106)

Data represent means ± SD ($n = 3$). % of spontaneous = [(No. of his^+ revertants in the presence of sample)/(No. of spontaneous revertants)] × 100. The number of spontaneous revertants was determined without samples and mutagens.

production of cytotoxicity. Fig. 2 reveals that AML could protect HepG2 liver cells against *t*-BHP induced oxidative damage and cytotoxicity. Thus, it seems that the protective effects of AML on the cytotoxicity of HepG2 cells induced by *t*-BHP may be due to the decrease of oxidative stress, consequently preventing *t*-BHP induced cytotoxicity in cells. On the other hand, as shown in Fig. 3, the *t*-BHP induced intracellular ROS production relates well to a clear depletion of GSH levels in HepG2 liver cells. GSH is known to function as a reductant due to its side chain sulfhydryl residue in the cysteine of GSH, which is the most important biomolecule against chemically induced cytotoxicity (Yuan & Kaplowitz, 2009). This residue can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction. However, once oxidative stress elevates in cells, the concentration of GSH is reduced, leading to the imbalance of the redox status of cells. In the present study, the level of GSH in HepG2 cells induced by *t*-BHP is obviously decreased. As seen in Fig. 3, AML not only reduce *t*-BHP induced ROS generation, but also prevent cell damage from glutathione depletion in HepG2 liver cells. These data imply that AML might offer protection against mutagens induced mutation and oxidative stress in liver cells.

Though a number of studies have suggested that some phytochemicals may exhibit mutagenic and cytotoxic activity (Ames, 1983), AML shows neither toxicity nor mutagenicity toward *S. typhimurium* TA 98 or TA 100 in the present study. However, *S. typhimurium* is a bacterium and thus not a perfect model of the human body. In fact, some substances that cause cancer in laboratory animals do not give a positive Ames test. On the other hand, the bacterial reverse mutation test is rapid, inexpensive and relatively easy to perform. We determine the highest amount of test substance to be used by cytotoxicity test. In this study, AML (1–5 mg/plate) did not show any cytotoxicity against TA 98 or TA 100. Therefore, the recommended maximum test concentration for non-cytotoxic substances is 5 mg/plate.

The mechanisms of antimutagenesis have different pathways, including deactivation of mutagens, inhibition of metabolic activation of promutagens and deactivation of activated mutagens. In this study, 4-NQO produced the ultimate mutagenic compound, which bound to DNA and generated stable quinoline–purine mono adducts (Arima et al., 2006). On the other hand, 2-AA preferred the biotransformation pathway, where a cytochrome P450-dependent monooxygenase produced a nitrene moiety that bound to DNA (Baker et al., 2001). Natural products contain many substances that can likely reduce mutation and cancer. For example, *Origanum vulgare* L. ssp. *Vulgare* (Gulluce et al., 2012) and sugarcane leaves also show an antimutagenic effect against different mutagens toward *S. typhimurium* mutation (Wang, Duh, et al., 2011). As shown in Table 3, AML played an antimutagenic role to suppress the mutagenicity of 4-NQO and 2-AA in the Ames test model. These data implied that the conjugated reaction between AML and the toxic electrophile was an important detoxification pathway. On the other hand, AML might play an antimutagenic role by scavenging the active metabolic electrophile of 4-NQO and 2-AA. The antimutagenic effects of the AML might also be attributable to decrease metabolic activation and the levels of toxic reactive intermediates, which further indirectly reduced cellular oxidative stress and, thereby, prevented mutation.

In summary, as mentioned above, AML demonstrates antimutation and antioxidation effects. These activities may be partially attributable to its phenolic constituents. Furthermore, the five marker compounds have similar activities to the extract that contain significantly lower levels of the five marker compounds. This suggests that other polyphenols or unknown active components (e.g. compounds **A**, and **B**) in AML could also play critical roles in its protective effects. Although these results demonstrate the protective effects provided by AML against mutation and oxidation, further investigations of the nutritional and physiological effects of AML are still required. However, the unknown compounds (e.g. compound **A**, and **B**) which were not positive identification have to be identified in our future study.

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