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RIPE *CLEISTOCALYX NERVOSUM* VAR. *PANIALA* BERRY FRUIT EXTRACT PREVENTS HYDROGEN PEROXIDE-INDUCED DNA DAMAGE IN HUMAN MONOCYTE U937 CELLS

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ABSTRACT

Intracellular reactive oxygen species (ROS) production and accumulation cause oxidative stress, leading to an increase in DNA damage, which is one of the major causes of mutation and diseases, especially cancer. Antioxidants in various fruits that play an important role in reducing ROS and oxidative stress. *Cleistocalyx nervosum* var. *paniala* (Ma-kiang: MK) is a native berry found in Northern Thailand which has strong antioxidants properties. The present study aimed to examine the preventive effect of ripe MK fruit extract on hydrogen peroxide-induced DNA damage in human monocyte U937 cells. Firstly, MK was extracted with 95% ethanol, then antioxidant capacities were evaluated. The results exhibited that MK extract was strongly able to scavenge radicals, especially the hydroxyl radicals, and had potent total flavonoid and phenolic contents. For the cell-based experiments, we found that pretreatment U937 cells with MK extract obviously increased cell viability and suppressed intracellular ROS in the cells induced by hydrogen peroxide. Furthermore, MK extract could significantly inhibit hydrogen peroxide-induced oxidative DNA damage in human monocyte U937 cell model. Thus, these results indicate that ripe MK berry extract might be utilized as an alternative antioxidant and geno-protective agent for reducing risk of oxidative stress and mutation-related diseases.

Keywords: antioxidant, *Cleistocalyx nervosum* var. *paniala*, DNA damage, human monocytes U937 cells, oxidative stress, anthocyanin, cyanidin-3-glucoside (C3G)

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Introduction

The rising incidence of non-communicable diseases (NCDs) in Thailand, including coronary heart disease, hypertension, diabetes, obesity, and cancer, highlights a critical public health challenge. A 2023 survey by the Ministry of Public Health of Thailand identified cancer as the leading cause of death.[1] This situation underscores the need for improved health initiatives focused on prevention and early detection to enhance the well-being of the population. One of the factors that increase the risk of these diseases is the environmental such as pollution, ultraviolet rays; especially lifestyle, stress, and poor diets. They essentially affect the increasing risk of these chronic NCDs, which also contribute to the formation of free radicals in the body.[2]

The white blood cells in the human body, especially the monocyte and macrophage, play a vital role in an immune system that is associated with developing various NCDs and chronic inflammatory diseases.[3] In these conditions, monocyte and macrophage are persistently activated, leading to the sustained production of reactive oxygen species (ROS). Monocytes exhibit susceptibility to DNA damage from various factors, such as ROS, specific chemicals, radiation, and others. This sensitivity is associated with a lower expression of DNA repair proteins, particularly those involved in base excision repair and double-strand break repair. If the damage is too severe, it can also trigger apoptotic cell death.[3-8] Therefore, prolonged and accumulation of ROS, along with the cytokines and chemokines produced by ongoing monocyte activation, can infiltrate neighboring cells and cause damage to DNA and other cellular components.[6,7] This sustained damage results in significant harm to normal tissues, contributing to tissue degeneration and the development of NCDs such as atherosclerosis, arthritis, bowel disease, neurodegenerative disorders, and cancer, *etc*.[4] Moreover, excessive ROS and oxidative stress cause DNA injury and lesions, particularly 8-oxo-guanine (8oxoG) and thymine glycol,[9,10] stimulate mispairing and the formation of non-instructive lesions, which result in genome instability and mutations, leading to cancer development.[5,11] Prolonged accumulation of free radicals effectively results in the destruction or change of cells. Normally, the cellular system has a mechanism to terminate free radicals with antioxidant systems. These systems are the endogenous and exogenous antioxidants pathways. The endogenous antioxidants are produced in cellular such as superoxide dismutase, catalase and glutathione peroxidase, and glutathione, *etc*.[12] While the exogenous antioxidants that come from outside the body. It obtained from various diets including vitamin C, vitamin E, phytochemicals such as flavonoids, carotenoids, phenolic acid, resveratrol, and anthocyanin, *etc*.[11,13,14]

Cleistocalyx nervosum var. *paniala*, a local plant found in the Northern region of Thailand, is called in Ma-kiang (MK). It is classified in Myrtaceae family that is one of Thai berry fruit.[15] Ripe MK berry is sour-sweet and slightly astringent, appearing red to dark purple, which is a characteristic pigment of an active compound in anthocyanin. Major anthocyanins in MK contain cyanidin-3-glucoside, cyanidin-5-glucoside, and cyanidin-3, 5-diglycoside.[16] Previously, the study reported that ripe MK fruit extract could increase the amounts of white blood cells, particularly the natural killer cells.[17] Moreover, MK extract prevents mouse hippocampal neuronal cell death.[18,19] A previous study showed the inhibitory effects MK fruit on mutagenesis using *Salmonella* assay and reduced micronuclei in liver of rat.[19] Research indicates that anthocyanins can significantly lower the risk of cardiovascular disease, diabetes, and cancer by harnessing their powerful antioxidant and anti-inflammatory effects.[20,21] Numerous studies have demonstrated that anthocyanins possess anti-carcinogenic properties characterized by several key mechanisms. These include the scavenging of free radicals, the stimulation of phase II detoxifying enzymes, the reduction of cancer cell proliferation,

the attenuation of inflammation, and the induction of apoptosis.[22] Several lines have shown that anthocyanin plays a role in suppressing cancer in various cell types and animal models.[23-25] The scientific information provided highlights a compelling reason to use local fruits known for their strong antioxidant properties. MK fruit is particularly rich in biologically active compounds, making it a suitable candidate for investigating preventive treatment for cell damage caused by free radicals.[15,23,26] Our research focused on the protective effects of such berries, while also emphasizing the development and utilization of local plant resources. Therefore, we aimed to investigate the antioxidant activities and antigenotoxic effect of ripe berry fruit extract of MK induced by hydrogen peroxide in human monocyte U937 cell line.

Materials and Methods

1. Chemicals and reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, sodium bicarbonate, fetal bovine serum, N-lauroylsarcosine phosphate anhydrate, ethanol, dimethyl sulfoxide (DMSO), and cyanidin-3-glucoside (C3G) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizma base, zinc sulfate and potassium dihydrogen phosphate were purchased from Fluka analytical (Munich, Germany). Ethylene diamine tetraacetic acid (EDTA), silver nitrate, Low melting point agarose (LMA), tungstosilicic acid, sodium hydroxide, sodium dihydrogen phosphate mono hydrate, and sodium chloride were purchased from Merck kGaA (Darmstadt, Germany).

2. Preparation and extraction of sample

The ripe MK fruit from Chiang Mai province were collected during July-August 2020. This plant was identified by Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand and its voucher specimen was BCU-A013732. The procedure of sample preparation and extraction was slightly modified from Nantacharoen et al.[18] Briefly, the ripe MK fruits were cleaned, and the pulp was separated from the seed. The sample was lyophilized and extracted with 95% ethanol (ratio of MK powder 1 g to 95% ethanol 10 mL) using Soxhlet extraction overnight at 50 °C. Next, the extracted solution was evaporated with a rotary vacuum evaporator at 40-50 °C until dry, according to previous studies.[18,27-29] The percentage of yield was 47.89±3.12%. After that, MK extract was dissolved in absolute DMSO then filtered through 0.2 µm pore that was used as a stock solution and stored at -20 °C until further investigation.

3. Evaluation of antioxidant activities of the MK extract

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Free radical scavenging ability of the MK extracts from different concentration of the samples were estimated by DPPH assay. MK extract (20 µl) at concentrations 1-100 µg/ml was added in the 96-well plate then added 200 µl DPPH solution and incubated in the dark for 30 min at room temperature. Trolox used as the standard solution. The absorbance was measured at 520 nm using a microplate reader (BioTek® Instruments, Vermont, USA). Results were calculated as % scavenging activity = [(absorbance of blank - absorbance of sample) / absorbance of blank] × 100 [18,23,30] and expressed as the inhibitory concentration $(IC)_{50}$ value by linear equation.

Ferric reducing antioxidant power (FRAP)

Reducing power of antioxidant were measured by ferric ion reducing antioxidant power assay. The antioxidant was reacted with a yellow ferric tripyridyltriazine complex change to ferrous Tripyridyltriazine, which is purple in color. The 20 µl of MK extract (100 µg/ml) and place it in a 96-well plate, then added 150 µl FRAP and incubated in dark for 8 min at room temperature, using trolox as a standard solution. The absorbance was measured at a 600 nm with a microplate reader (BioTek® Instruments, Vermont, USA). The absorbance values

were calculated with the standard solution graph, the results are expressed as mmol of trolox equivalent (TE) per gram of dry weight (DW) sample.[18,23,30]

Total phenolic content

The Folin–Ciocalteu assay was used to measure total phenolic content, which helps reduce oxidation reactions based on the Folin–Ciocalteu colorimetric method,[18] MK extract was performed by absorbing or binding to free radicals or neutralize of the test substance by react the substance with Folin-Ciocalteu reagent. MK extract (10 µl) at 100 µg/ml was added into a 96-well plate and then added 160 µl of distilled water followed by 10 µl Folin-Ciocalteu solution and 20 µl Na_2CO_3 solution, then incubated in the dark for 30 min at room temperature. Gallic acid solution was used as a standard solution. Microplate reader (BioTek® Instruments, Vermont, USA) were used at the absorbance 750 nm. Results are expressed as gallic acid equivalents (GAE) in mg per gram of DW sample.

Total flavonoid content

The flavonoid content was evaluated by the aluminium trichloride method using quercetin as reference compound. MK extract (25 µl) at 100 µg/ml was added to 75 µl of 90% ethanol in a 96-well plate and followed by 5 µl of 10% aluminium trichloride, then gently mixed, added 5 µl of potassium acetate. The mixture solution was adjusted to 250 µl with distilled water in the final volume, then incubated for 30 min. The absorbance was measured at 415 nm by microplate reader (BioTek® Instruments, Vermont, USA). The total flavonoid content was expressed as µg quercetin equivalents (QE) in mg per gram of DW sample.[31]

Hydroxyl radical (OH•) scavenging activity

This assay was used to evaluate the scavenging hydroxyl radical activity, which is a crucial antioxidant activity due to the extremely high reactivity of OH radicals, leading to the reaction and damage of the various biomolecules and cells of the organism. This radical can be generated by H_2O_2 and the Fenton reaction.[32] Briefly, the reaction mixtures, which contain the 200 µl of 100 mM KH_2PO_4/KOH buffer pH 7.4, 200 µl of 16.8 mM of deoxyribose, 200 µl of the extract at concentrations 1-100 µg/ml or standard solution (trolox), 200 µl of 500 µM $FeCl_3$, 100 µl of 1.2 mM EDTA, and 100 µl of 1 mM ascorbic acid, followed by 200 µl of 10 mM H_2O_2, were mixed well. After incubation at 37°C for 1 h, one milliliter of 1% TBA and 1% TCA was added to the mixture, which was then incubated at 90 °C for 20 min. The intensity of the yellow color form was measured at 532 nm using microplate reader. The results are calculated as percentage of inhibition and IC50 value.

4. Cell culture

The human monocyte U937 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and used as an experimental model. The cells were cultured in complete RPMI 1640 medium supplement with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a moistened atmosphere containing 5% CO_2 and 95% air. Cells were passaged after 6-7 days with about 80% confluences.

5. Cell viability test

Measurement of cytotoxicity of the extract and of hydrogen peroxide treatment were evaluated by 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide or MTT method. It is colorimetric measurement of MTT that reduced by mitochondrial reductase in mitochondria to change the color of MTT from yellow to purple of formazan.[17,18] The human monocyte U937 cells were seeded overnight on 24-well plates at a density of 500,000 cells/well. To assess the cytotoxicity of MK extract and choose the non-toxic concentrations, cells were treated with different concentrations (1-1,000 µg/ml) of the extract or C3G (1-100 µM) for 24 h. For the preventive effect, the cells were pretreated with MK extract in various concentrations (1-100 µg/ml) for 24 h and exposed with hydrogen peroxide at 200 µM for 1 h. In addition, the final concentration of DMSO was kept equal at 0.2% in all extract-treated samples and in the cell

control, which also served as the vehicle (solvent) control. This was done to ensure that any observed effects on cell viability was attributed to the MK extract itself and not to the solvent. Next, fifty microliters of MTT solution at a concentration of 0.5 mg/ml was added to each well. After incubation 4 h at 37°C, the supernatant was removed and then DMSO was added to dissolve the formazan crystals. The plate was measured on a microplate reader (BioTek® Instruments, Vermont, USA) at 540 nm. Results are expressed as the percentage of cell viability calculated relative to the control cells.

6. Determination of intracellular ROS

The monocyte U937 cells were seeded overnight on 24-well plates at a density of 500,000 cells/well. Cells were treated with MK extract at various concentrations (1-100 µg/ml) or C3G (10 µM) for 24 h alone or followed by hydrogen peroxide at 200 µM for 1 h. Then, DCFH-DA (200 µl) at 5 µM was added in each well for 30 min at 37 °C. After incubation, the cells were washed, trypsinized, and resuspended in buffer. Fluorescent intensity was measured using a microplate reader (BioTek® Instruments, Vermont, USA) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Results are presented as relative fluorescence intensity.

7. **Measurement of genotoxicity in U937 cells by comet assay**

The comet assay or DNA damage analysis or single cell gel electrophoresis (SCGE) was performed as described by Sukprasansap and colleagues (2019).[33] Briefly, the U937 cells were treated with MK extract (1-100 µg/ml) or C3G at 10 µM for 24 h, followed by hydrogen peroxide at 200 µM for 1 h, then harvested and diluted with PBS to $4x10^5$ cells/ml. Cells (100 µl) were combined with 1% (w/v) low melting point agarose in PBS 100 µl at 37 °C and mixed gently, followed by dropping 75 µl onto a slide pre-coated with 1% normal melting point agarose and covering it with a coverslip until the gel solidified on ice for 15 minutes, then the coverslip was removed. Next, the slides were carried out to place in coplin jar containing lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, NaOH (pH 10), 1% N-Laurylsarcosine sodium, 1% Triton X-100, and 10% DMSO) at 4 °C. After incubation for 1 h, the slides were placed in an electrophoresis tank containing alkaline electrophoresis buffer (pH 13) at 4°C for 40 min. Electrophoresis was then performed at 300 mA and 25 V for 20 min. Afterward, the slides were washed with 0.4 M Tris-HCl buffer to neutralize the pH, followed by distilled water, and placed the slide to dry at room temperature overnight. Subsequently, the slides were stained with silver staining that DNA strand breaks were detected by light microscope (Nikon microscope, ECLIPSE E100, USA). The damage levels of DNA were observed by breaking down DNA, which is like a comet, using comet visualization and scoring. One hundred DNA cells were scored from each slide. DNA damage was classified into five levels: class 0 (no DNA damage), class 1 (low DNA damage), class 2 (moderate DNA damage), class 3 (high DNA damage), and class 4 (severe DNA damage),[34] as represented in Figure 1. The results were calculated by arbitrary unit (AU) and expressed as the percentage of DNA damage, following formula where AU is the total score of 100 cells damaged; The AU = 0(n lever 0) + 1(n level 1) + 2(n level 2) +3(n level 3) +4(n level 4) × 100] / total comets analyzed; % DNA damage = (AU/400) x 100.[34-36]

8. Statistical analysis

All results are presented in the mean ± standard deviation (SD) in three independent experiments. Statistical analysis was performed using SPSS software, version 22.0. One-way analysis of variance (ANOVA) was conducted, followed by Scheffé's post hoc test for multiple comparisons. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

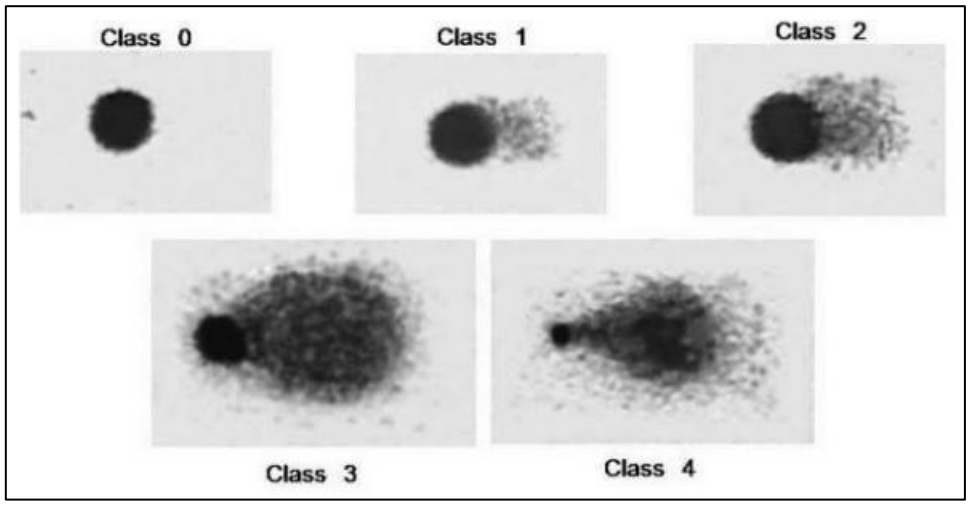

Figure 1 Classification of DNA damages[34]

Results

1. Antioxidant activities, total flavonoid and phenolic contents of MK extract

Antioxidant properties and total bioactive compounds of MK extract including radical scavenging activities, reducing power, total phenolic and flavonoid contents were evaluated. The results showed that the IC_{50} value of DPPH radical scavenging activity of MK extract was equal to 21.92±4.08 µg/ml (% radical scavenging activity was 70.58±6.07). Trolox, which was used as a reference compound, had an IC_{50} value of 0.79±1.18. The MK extract had a high ability to scavenge the DPPH radical, whereas the extract had a low reducing power according to the FRAP assay (Table 1). Interestingly, MK extract exhibited a strong inhibitory effect against hydroxyl radicals, with an IC_{50} value of 3.08±2.05 mg/ml (% inhibition was 95.37±6.32). For the total bioactive compound testing, MK extract at a concentration of 100 µg/ml exhibited a total phenolic content of 567.75±6.85 mg GAE/g DW and a total flavonoid content of 109.65±25.16 µg QE/g DW (Table 1). Furthermore, to demonstrate the correlation between antioxidant capabilities and the total bioactive compound contents of MK extract, all correlation coefficients were summarized as shown in Table 2. Our results showed that DPPH had a high relationship with hydroxyl radical scavenging activity and FRAP, with R values of 0.834 and 0.795, respectively. FRAP had a relationship with total phenolic content and total flavonoid content, with R values of 0.899 and 0.856, respectively. In addition, total phenolic content was also related to total flavonoid content at the level of R of 0.943 (Table 2).

2. Effect of MK extract on cell viability in U937 monocyte cells

The cytotoxicity of MK extract was accessed by cell metabolic activity using MTT assay. In this study, the U937 cells were treated with MK extract at various concentrations (1-1,000 µg/ml) for 24 h. The results showed that MK extract concentrations ranging from 1 to 100 µg/ml did not exhibit cytotoxic effects which their percent of viability of the cells were higher than 80%, compared to cell control (Figure 2). In addition, the vehicle control (cell control group) with 0.2% DMSO showed no effect on cell viability. While concentrations exceeding 100 µg/ml significantly decreased cell viability, which was lower than 80% compared to the cell control. Consequently, the maximum concentration of MK extract at 100 µg/ml was selected for subsequent investigation.

Table 1 Antioxidant capabilities of MK extract

Trolox IC_{50} value (µg/ml)	DPPH IC_{50} value (µg/ml)	FRAP (mmole TE/g DW)	Hydroxyl radical scavenging activity IC_{50} (µg/ml)	Total flavonoid content (µg QE/g DW)	Total phenolic content (mg GAE/g DW)
0.79±1.18	21.92±4.08	5.59±0.21	3.08±2.05	109.65±25.16	567.75±6.85

Values are the mean ± SD (n = 3). The concentration of MK extract was used as 1-100 µg/ml for IC50 value and 100 µg/ml for others. Dry weight (DW); Trolox equivalent (TE); Quercetin equivalent (QE); Gallic acid equivalent (GAE)

Table 2 Correlation coefficients between DPPH, FRAP, total phenolic content, total flavonoid, and hydroxyl radical scavenging activity of MK extract

	Correlation coefficient (R)				
	DPPH	FRAP	Total phenolic content	Total flavonoid content	Hydroxyl radical scavenging activity
DPPH	1				
FRAP	0.795	1			
Total phenolic content	0.576	0.899	1		
Total flavonoid content	0.501	0.856	0.943	1	
Hydroxyl radical scavenging activity	0.834	0.637	0.414	0.370	1

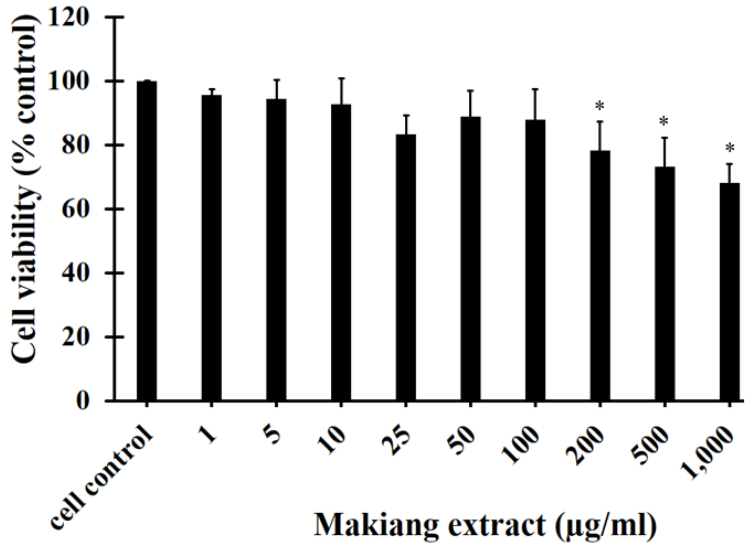

Figure 2 Effect of makiang extract on cell viability in U937 cells. Cell viability was determined by MTT assay. Cells were treated with MK extract (1-1,000 µg/ml) for 24 h. Data were presented as the percentage of cell viability. Values are the mean ± SD (n =3). * $p < 0.05$ versus the cell control.

3. Effect of MK extract on H_2O_2-induced oxidative toxicity in human monocyte U937 cells

The pretreatment cells with MK extract were used for the evaluation of the effect of MK extract on cell viability in U937 monocyte cells induced oxidative stress with H_2O_2. In the preliminary study, we examined the effect of H_2O_2 on cell viability at various concentrations for different times. Our data found that a concentration of 200 µM H_2O_2 for 1 h caused a significant reduction of cell viability by about 50% (52.39±3.87%) in U937 cells compared to cell control (data not shown); therefore, the H_2O_2 at 200 µM for 1 h was chosen for this experiment. The U937 monocyte cells were pre-treated with MK extract at three concentrations (1, 10, and 100 µg/ml) for 24 h, followed by 200 µM of H_2O_2 for 1 h. We found that all three concentrations of extract slightly decreased cell viability but were not toxic to cells (the cell viability was more than 80%) compared with the control group (Figure 2). In the absence of MK extract, H_2O_2 significantly decreased the viability of cells by 53.85±1.82%, compared to the non-treated control cells. Pretreatment cells with MK extract significantly attenuated the toxicity induced by H_2O_2 by increasing cell viability to 72-76%, compared with H_2O_2-induced cells group (Figure 3). Thus, the results suggest that the preventive effect of MK extract against oxidative toxicity induced by H_2O_2 in this U937 monocytes system.

4. Effects of MK extract on H_2O_2-induced intracellular ROS production in human monocyte U937cells

It is widely established that H_2O_2 is a major mediator in generating ROS in cellular systems. Thus, we whether examined the inhibitory effect of MK extract on oxidative stress caused by H_2O_2 in U937 cells. The ROS level in cells was measured by DCFH-DA fluorescence probe. Cyanidin-3-glucoside or C3G at 10 µM used as positive control in our cell model. The C3G was evaluated various concentrations of C3G (1-100 µM) in U937 cells. Results showed that the highest concentration of 10 µM C3G was non-toxic and did not affect the cell viability of U937 cells compared to the control group ($p < 0.05$); therefore, this concentration of C3G at 10 µM was chosen for this experiment (data not shown). The U937 cells were pretreated with MK extract (1, 10, and 100 µg/ml) for 24 h, followed by 200 µM of H_2O_2 for 1 h. We first evaluated the effect of treating MK extract or C3G alone on ROS production in U937 monocyte cells. Results showed that all concentrations of MK extract and C3G could reduce the intracellular ROS production in normal conditions within the cells compared to the control group (Figure 4A). In addition, the H_2O_2 treatment significantly increased the intracellular ROS generation by 2.27±0.27 fold, compared to the untreated control. Pretreating cells with MK extract significantly inhibited the ROS caused by H_2O_2; meanwhile, C3G treatment also suppressed the ROS, similar to the MK extract treatment (Figure 4B). Therefore, the results demonstrate that pretreatment of cells with MK extract reduces the intracellular ROS production caused by H_2O_2 in human monocyte U937 cells.

5. Effect of MK extract on H_2O_2-induced DNA damage in human monocyte U937 cells

Since results above reveal that MK extract could inhibit ROS which involves in reducing the accumulation of oxidative stress in cells. Thus, we further elucidated the protective effects of MK extract against H_2O_2-mediated oxidative stress and DNA damage in U937 human monocytes using DNA damage-single cell gel electrophoresis analysis or the comet assay. The U937 cells were pretreated with MK extracts or C3G for 24 h, followed by 200 µM of H_2O_2 for 1 h. Figure 5A shows that the treatment of U937 cells with MK extract or C3G alone exhibited a slight percentage of DNA damage, which they were classified into class 1 (low DNA damage) while the control group was classified into class 0 (no DNA damage).

Obviously, the H_2O_2-treated cells significantly increased the DNA damage by approximately 85% compared with the untreated control group (Figure 5B),

which are classified as severe DNA damage in class 4. Interestingly, pretreating U937 cells with all concentrations of MK extracts or C3G alone remarkably suppressed H_2O_2-induced DNA damage compared to H_2O_2 treatment alone (Figure 5B). The pretreatment cells with MK extracts at 1 and 10 µg/ml were classified as class 2 with moderate DNA damage, whereas 100 µg/ml of MK extract and 10 µM C3G were indicated in class 1 (low DNA damage) to reduce DNA damage caused by H_2O_2.

Especially, MK extract at 100 µg/ml was shown to be highly effective in reducing DNA damage, similar to the C3G treatment group, compared with H_2O_2-treated cells group (Figure 5B). Our results indicate that H_2O_2 treatment has the potential to cause oxidative toxicity and DNA damage, while MK extract can inhibit this DNA damage of human monocyte U937 cells induced by H_2O_2.

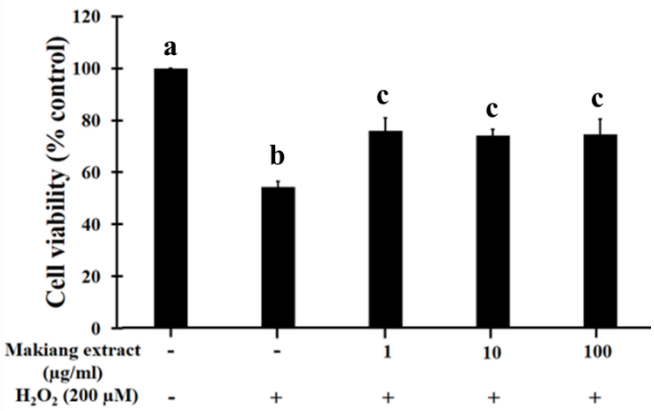

Figure 3 Effect of makiang (MK) extract on H_2O_2-induced toxicity in human monocyte U937 cells. Cells were pretreated with MK extract (1, 10, and 100 µg/ml) for 24 h, followed by 200 µM of H_2O_2 for 1 h. Results are expressed as the mean ± SD (n=3). Columns with different superscript letters were significantly different ($p < 0.05$). Different letters (a-c) indicate significant differences among groups with $p < 0.05$.

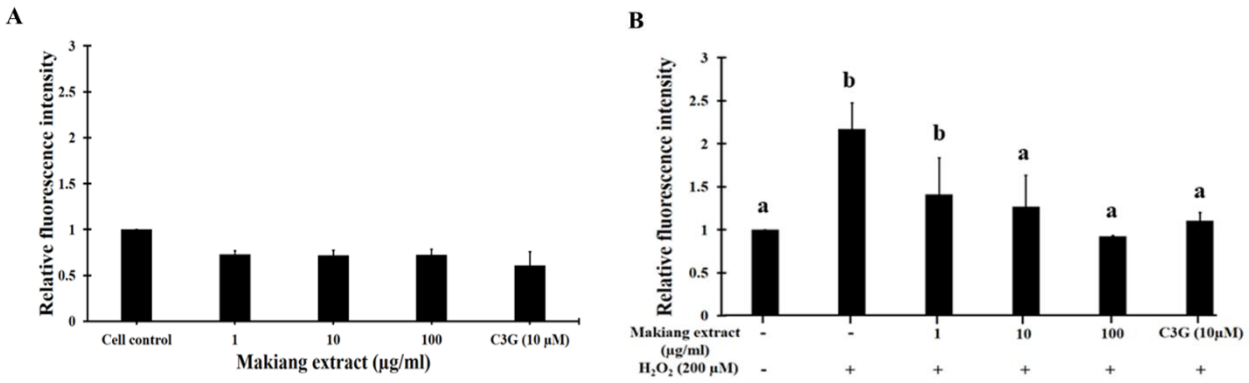

Figure 4 Effect of MK extract on H_2O_2-induced ROS in U937 cells. (A) Cells were treated with MK extract (1, 10, and 100 µg/ml) or C3G (10 µM) for 24 h without H_2O_2. (B) Cells were pretreated with MK extract (1, 10, and 100 µg/ml) or C3G (10 µM) for 24 h, followed by 200 µM of H_2O_2 for 1 h. Data are presented as the relative fluorescence intensity compared to the untreated control. Values are expressed as the mean ± SD (n = 3). Columns with different superscript letters were significantly different (p < 0.05). Different letters (a-b) indicate significant differences among groups with $p < 0.05$.

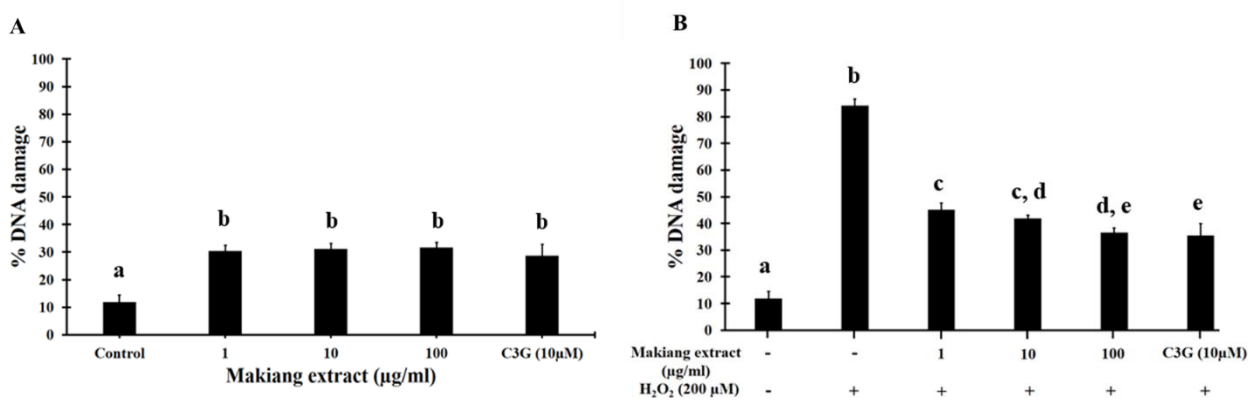

Figure 5 MK extract reduces DNA damage in human monocyte U937 cells induced oxidative stress by H_2O_2. (A) Cells were treated with MK extract (1, 10, and 100 μg/ml) for 24 h without H_2O_2. (B) Cells were pretreated with MK extract (1, 10, and 100 μg/ml) for 24 h, followed by 200 μM of H_2O_2 for 1 h. Bar graphs represent as %DNA damage. Values are the mean ± SD (n = 3). Columns with different superscript letters were significantly different ($p < 0.05$). Different letters (a-e) indicate significant differences among groups with $p < 0.05$.

Discussion

Overproduction and prolonged accumulation of ROS are related to the development of many human diseases including NCDs, which are associated with several acute and chronic pathological processes, such as cardiovascular diseases, neurodegenerative diseases, macular degenerative diseases, inflammatory-related diseases, and cancer.[2,3] In normal condition, free radicals occur in the body and are formed as by-products of regular metabolic cell activity, but if the body has an imbalance of free radicals and antioxidant, leading to the oxidative stress condition.[3,4] This event affects to the injury of cell membrane, protein, fat, and DNA, which contribute to the structure changes, loss of their normal functions, and cell death.[2-4,37,38] According to scientific reports, oxidative stress can cause DNA damage leading to genetic mutation.[38,39] Previous study reported that ROS directly stimulate DNA damage by oxidizing nucleoside bases.[39] The 8-hydroxy-7,8-dihydroguan-8-yl radicals generated by added hydroxy radical to C8 of the guanine base,[40] which can lead to G-T or G-A transversions.[41] Several studies found that oxidative stress reduces the growth of white blood cells and also increases the DNA damage.[42,43] Moreover, a decrease in immune function can also affect the removal of free radicals in the body, contributing to cell damage and susceptible to disease. In consequence, it may play an important role in the carcinogenesis and distribution of cancer cells.[44] Free radicals have many forms, such as superoxide anion radical, hydroxyl radical and non-radicals such as hydrogen peroxide.[45] Although the hydrogen peroxide is not the form of radicals, but it can rapidly oxidize to hydroxyl radical form which has a significant effect on DNA in cells.[46] Exposure of U937 human monocytic cells to H_2O_2 induces significant oxidative DNA damage primarily through hydroxyl radical generation. This oxidative stress is further exacerbated by the formation of singlet oxygen, a highly reactive species that amplifies cellular injury. The extent of DNA damage caused by ROS can be sensitively assessed using the comet assay, which quantifies DNA strand breaks based on the length and intensity of comet tails at the single-cell level. Concurrently, the antioxidant potential of bioactive compounds against free radicals can be evaluated through standard antioxidant assays, including DPPH, FRAP, hydroxy radical scavenging, *etc*. These methods provide quantitative insights into a compound's capacity to scavenge free radicals and

mitigate oxidative stress.[6] Human monocytic U937 cells were selected as the model for evaluating antioxidant and antigenotoxic activities due to their well-established sensitivity to oxidative stress and ROS. These cells serve as a widely used model to investigate the cellular response to oxidant-induced DNA damage, particularly caused by H_2O_2 which generates highly reactive hydroxyl radicals and singlet oxygen within the intracellular environment.[7] The U937 cell line also offers practical advantages in *in vitro* assays due to its reproducibility, responsiveness to pro-oxidants, and compatibility with standard ROS detection and genotoxicity assays, such as the comet assay and dot blot.[6-8,47] Furthermore, previous studies have demonstrated that phytochemicals can modulate ROS levels and DNA integrity in U937 cells, confirming their suitability for assessing both oxidative injury and protective effects of antioxidant compounds.[6,8]

Edible plants play a role as an antioxidant in the protection of free radicals. Many natural antioxidants have been examined in the *in vitro* or *in vivo* models to assess their potential preventive and therapeutic effects in conditions linked to oxidative stress-mediated cytotoxicity. Numerous vegetables and fruits and their active compounds dominate the powerful antioxidants that are necessary for the prevention and treatment of various NCDs.[37,47] Therefore, the search for natural antioxidants from plants that effectively inhibit ROS generation or directly interact with ROS produced by hydrogen peroxide remains ongoing research. Particularly, the natural antioxidants from Thai local berries are likely to inhibit hydrogen peroxide-mediated ROS and DNA damage in white blood cells, is few data. Some study showed that Thai berries possess antioxidant activity due to high anthocyanin content, mainly cyanidin derivatives. In *Prunus domestica* L., *Antidesma bunius* L. Spreng, *Syzygium cumini* Skeels, and *Syzygium nervosum* A. Cunn., phenolics, flavonoids, and anthocyanins ranged from 222.7–283.5, 91.2–184.3, and 37.9–49.5 mg/g extract, respectively.[48] Thus, in the present research, we aimed to focused on the antigenotoxic effects of *C. nervosum* var. *paniala* or Makiang fruit extract on oxidative toxicity and DNA damage induced by H_2O_2 in human monocytes.

C. nervosum var. *paniala* or Makiang is one of the local Thai berries found in the North of Thailand, which was used in this study. Its ripe fruit berry possesses antioxidant properties, which have been revealed to promote health benefits through a wide range of biological effects. Ripe MK fruit contains anthocyanins, tannin, rutin, and resveratrol.[16,18,19,23,26,30] These substances belong to the active ingredient group of polyphenol compounds that plays an important role in antioxidant.[17] This compound has anti-inflammatory and anti-cancer effects.[14] Previous studies have shown that MK extract had potent anti-mutagenic effects in both *in vitro* and *in vivo* researches.[14,17,19] Some study found that MK extract reduced the genetic toxicity in the bacterial mutation assay.[19] In addition, MK fruit extract inhibited oxidative kidney damage by cadmium in rat,[49] and suppressed endoplasmic reticulum stress-induced hippocampal neuronal cell death.[23] The MK fruit extract could stimulate the lymphocyte proliferation and enhance the function of white blood NK cells.[17] Our present study found that ethanolic extract of ripe MK fruit showed the powerful antioxidant capabilities through the direct scavenging property by DPPH radical and hydroxyl radical scavenging activities (Table 1). These are involved in the high contents of total phenolics and total flavonoids of the extract, consistent with several studies.[17,18,23,30,31] The previous studies reported lower or higher values of DPPH radical scavenging activity, total flavonoid content, and total phenolic content in *C. nervosum* var. *paniala* fruit extracts compared to the present findings. These differences may be attributed to seasonal variations and geographical factors, which are known to significantly influence the antioxidant properties and phytochemical composition of the plants, including *C. nervosum* var. *paniala*.[16,18,19,23,26,30,31,49,50] In addition, we

evaluated the hydroxyl radical scavenging activity of the extract since this radical was generated by hydrogen peroxide, which was used as a mediator for inducing oxidative toxicity in our experimental cell model. Our results demonstrated that MK extract can distinctly inhibit hydroxyl radicals. According to the correlation of several antioxidant activities of MK extract, we found that the high level of flavonoids was associated with the high amounts of phenolic compounds because the flavonoids were a sub-class of the phenolic compound groups, resulting in the R value being nearly one (Table 2). Also, the correlation coefficient of DPPH was linked to the hydroxyl radical activity since their reactions were the scavenging effect, resulting in the R value being nearly one, as shown in Table 2. Therefore, it is possible that this ripe MK fruit extract has the potential to be used to further inhibiting oxidative cytotoxicity.

Next, to examine the protective effects of MK fruit extract on hydrogen peroxide-induced oxidative toxicity and DNA damage, we used the human monocyte U937 cells as a model in this experiment. Firstly, we verified the cytotoxicity of MK extract. Our results exhibited the maximum concentration of MK extract at 100 µg /ml had no cytotoxic effect (Figure 2). While the higher concentrations of the extract showed the cytotoxic effect and decreased the viability of cells. Although, MK extract possesses a polyphenol agent that revealed antioxidant properties in normal condition, but excessive dose of this extract could be changed in oxidant effect property known as the pro-oxidant toxic effects.[51-53] Additionally, we observed the effect of hydrogen peroxide on cell viability and ROS production in U937 monocyte cells. The treated cell with hydrogen peroxide at 200 µM for 1 h reduced cell viability about fifty percent (Figure 3). This result related to produce the ROS in the U937 cells (Figure 4). It elucidated that hydrogen peroxide significantly generated intracellular ROS and induced oxidative toxicity to our cell model. Furthermore, the results demonstrated that treating U937 cells alone with all concentrations of MK extract (1-100 µg/ml) and its active component, C3G (10 µM), did not generate intracellular ROS, compared to the control group (Figure 4). Remarkably, our protective effects results showed that pretreatment of cells with MK extract clearly suppressed H_2O_2-induced intracellular ROS generation and increased cell survival in U937 cells (Figures 2 and 3). Consequently, this pretreatment model was further investigated using the comet assay to demonstrate the antigenotoxic effect of MK extract on H_2O_2-induced DNA damage in U937 human monocytes. Our results also indicated that MK extract could protect against hydrogen peroxide-induced DNA damage (Figure 5). Interestingly, MK extract at 100 µg/ml demonstrates a significant ability to suppress H_2O_2-induced DNA damage, showing results similar to those of its active component, C3G treatment group. This suggests the potential for MK extract in DNA protection strategies. Although results showed that the extract treatment induced DNA damage but all of them were classified as class 1 of DNA damage at a low level. They depend on the classification of DNA damage level and are involved in the DNA repair system within cells. In addition, this event might be related to the oxidation reaction, resulting in injury to the double-strand break DNA.[3-5,9,10] This insight underscores the need for further investigation into the implications of such damage on overall cellular health. Excessive production of ROS can cause damage to cellular components, especially DNA, proteins, and lipids. This occurrence promotes carcinogenesis by inducing DNA damage and genetic mutations.[54,55] Several studies showed that hydrogen peroxide obviously induced ROS generation, and oxidative stress, leading to DNA fragments and cell death, while treatment with N-acetyl cysteine (a well-known antioxidant agent) inhibited oxidative stress-induced mitochondrial dysfunction and cell death.[56,57] Previous research found that the MK fruit extract reduced ROS production and cell death in various cells.[18,23,30] In addition, MK fruit extract was also observed to reduce the level of ROS inside the nematode during oxidative stress.[50] Moreover, the

protective effect of MK fruit extract that not only in the direct scavenging property, but the extract also improved and enhanced the gene expression of cellular antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, that were observed in mouse hippocampal neuronal cells.[18,23] Furthermore, MK fruit extract can stimulate antioxidant mechanism via Nrf2 pathway leading to reduce intracellular ROS levels and oxidative stress,[23,50] and also increase the lifespan of *Caenorhabditis elegans*.[31,50] In addition, a previous data reported that the MK seed extract enhanced the function of heme oxygenase-1 and attenuated oxidative stress in rat liver.[19] Thus, MK extract plays an important role in the protective response to oxidative toxicity. Accordingly, this genoprotective effects were involved to the powerful antioxidant capability of the MK fruit extract. It might be an alternative promising functional food to use for preventing or inhibiting risk of genotoxicity-related disorders such as cancer. Moreover, it might be associated with many bioactive compounds that can be found in ripe MK fruit, especially major anthocyanins such as C3G[16,23] which play a critical role in reducing the production of ROS, contributing to the destruction of DNA through oxidizing nucleoside bases process.[39] Besides, some study found that dietary anthocyanin-enriched purple-fleshed sweet potato clone P40 had a protective effect against colorectal cancer by inducing cell-cycle arrest, anti-proliferative, and through apoptotic mechanisms in the colons of female CF-1 mice and also revealed a greater expression of apoptotic caspase-3 in the colon mucosal epithelial cells.[58] Another study also showed that the dietary anthocyanins can be attributed to the regulation of redox enzymes through reducing ROS production and modulation of the phase-II detoxifying enzymes responsible for the cellular oxidative response.[59] Although the present study does not establish the mechanism of the protective effects of MK extract; however, it demonstrates the extract's role in reducing oxidative toxicity and preventing DNA damage. Further research is necessary to explore the mechanistic aspects, such as the stimulation of endogenous antioxidant enzymes by MK fruit extract, to clarify the signaling pathways involved in cell death and cellular antioxidant activity of human monocyte U937 cells model, as well as to understand how the extracts may contribute to lowering the risk and mortality associated with this oxidative toxicity and DNA damage-related diseases.

Conclusions

Our study reveals that the preventive effects of *C. nervosum* var. *paniala* or MK berry fruit extract on H_2O_2-induced oxidative toxicity and DNA damage in human monocyte U937 cells. The results exhibited that MK extract has a strongly antioxidant capabilities and phenolic content. It is powerful able to scavenge the radicals especially hydroxy radicals caused by the hydrogen peroxide mediator that was used as inducer in our cell model. Notably, pretreatment of U937 cells with MK extract and C3G showed the improving of cell viability and decreasing of ROS production induced by H_2O_2. Interestingly, pretreated cells with MK extract or its bioactive substance-C3G could prevent oxidative DNA damage in response to H_2O_2. Our findings indicate that the Thai local MK berry has strong antigenotoxic properties, highlighting its potential for development as a valuable functional Thai berry.

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Conflict of Interest

The authors declare no conflict of interest.

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