

PHYTOCHEMICALS AND ANTIOXIDANT PROPERTIES OF WAN KHAN MAK (*Aglaonema simplex* BL.) FRUIT EXTRACT

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Abstract

Aglaonema simplex BL. (also called *wan khan mak* (WKM) in Thai), a plant in the family Araceae, has long been claimed in Thai traditional medicine to possess several ethnopharmacologic properties. Nevertheless, research into its claimed biological activities has never been explored. The present study aimed to investigate the proximate composition, the phytochemicals, and the chemical and intracellular antioxidant activities of 95% ethanol crude extract of the dried fruits of WKM. The results showed that the yield of WKM crude extract was 12.65% of the dry weight, and the contents of total phenolics and flavonoids in the extract contributed to the antioxidant capacities as demonstrated by DPPH and FRAP assays. The WKM extract could also effectively attenuate intracellular reactive oxygen species (ROS) generation in *t*BuOOH-induced oxidative stress in macrophage RAW264.7 cells, as monitored by using a DCFH-DA fluorescent probe. Overall, the present data suggest that WKM could be a potential source of natural antioxidants and merits further investigation for its possible roles in prevention and/or alleviation of ROS-mediated diseases.

Keywords: *Aglaonema simplex* BL., phytochemicals composition, DPPH assay, FRAP assay, DCFH-DA, RAW264.7 cells

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Introduction

Despite a bloom in research of Thai herbs in recent years, there is little research into *Wan*, the general name for sedges or orchids, compared with other herbs. Traditional Thai medicine textbooks have mentioned that most, if not all, of *Wan* are herbs which possess many medicinal and superstitious effects, including relief from the poisonous stings of insects and a disease cure, drug detoxification, general healing, warding off snakes, protecting against enemies, scaring away the devil, and for use in amulets for good luck in commerce (Sanyasee, 2018). *Wan* has been categorized into 34 families, 512 genera, and over 1,700 species. There are many interesting species of *Wan* and 1 of those is *Aglaonema simplex* or *wan khan mak* (WKM) (Figure 1). Thai-traditional medicine has cited WKM as a miracle plant that possesses many ethno-medical properties such as anti-aging, firming the skin, strengthening teeth, grey hair prevention, and as an energy booster (Sanyasee, 2018).



Figure 1. Fruits of *Wan Khan Mak*

At present, most people have not well realized the pharmacological values of WKM. It has been planted only for decoration or as plant for amulets to dispel bad luck, or attract good luck and fortune. So far, the study of WKM's pharmacological properties is still very limited. Panyaphu *et al.* (2011) surveyed 168 species of medicinal plants and reported that a poultice prepared from WKM leaves and

stems, among other preparations of medicinal plants, has anti-pain and anti-inflammation values in primary healthcare of postpartum women. Chee *et al.* (2005) found 5 pheophorbide-related compounds from the leaves and stems of WKM. All of them showed moderate to strong phytotoxicity towards several human cancer cell lines (HL 60, HSC-2, and HSC-3). To the best of our knowledge, the therapeutic properties and toxicity of the WKM fruits have never been reported. Although there is still no scientific information to support the biological activities of WKM fruit, people have recently become increasingly aware of its therapeutic values. Those who have consumed WKM fruit have claimed that it promoted good health, refreshed feelings, relieved an aching body, energized the body and produced a juvenile skin complexion. Nowadays, WKM fruits are being sold expensively on several websites ranging from 800- 1,000 baht per kilogram. Part of the reason for the price might be that the plant is rarely found, and it might not bear fruit every year if grown under inappropriate weather or conditions (Sangneam, 2018).

At present, WKM is a plant that has been selected as an affiliate in the Plant Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chari Sirindhorn (RSPG) (Soontornyatarata *et al.*, 2017). The objective of the project is to collect the genetic analysis and information on the pharmacological activities and bioactive compounds of traditional medicinal plants in Thailand. Although WKM has gained a reputation for its therapeutic values and is 1 of the selected plants under the RSPG project, there is still no research or any scientific evidence to support the claims for WKM's ethno-medical properties.

This research aimed to explore the chemical and phytochemical constituents of the ethanolic fruit extract of WKM and to investigate the antioxidant activities of the extract by using 3 different methods, namely the DPPH, FRAP, and DCFH-DA assays. This study only focuses on the WKM fruit as it is the part used in Thai traditional medicine and

is claimed to possess the most biological activity in the whole plant.

Materials and Methods

Chemicals and Reagents

The 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), and *tert*-butyl hydroperoxide (*t*-BuOOH) used in our research were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI medium 1640, MEM medium, and Hank's balanced salt solution (HBSS) were from Gibco Invitrogen (Grand Island, NY, USA).

Cell Lines

The mouse macrophage cell line (RAW264.7) gamma NO(-) (ATCC, CRL-2278) was used for the cellular antioxidant activity assay. RAW264.7 cells were cultured at 37°C, 5% CO₂ in humidified air and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin. Exponentially growing cells were used for the experiments when they reached approximately 80% confluence.

Plant Materials

The whole ripe fruits of WKM were harvested during December to January, the peak season of ripening, from Amphur Pakthongchai, Nakhon Ratchasima province, Thailand. The plant was identified and authenticated by the Forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen (BKF 186333) was deposited at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand.

Plant Extract Preparation

The fruits of WKM were dried at 40°C in a hot-air oven (Mettler GmbH & Co. KG, Schwabach, Germany), ground into powder, and then extracted by maceration in 95% ethanol for 48 h at room temperature, the condition providing the maximum yield from the preliminary study. After centrifugation,

the extract was concentrated by a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) and dried by lyophilization (Freeze-Zone 12 plus, Labconco Corporation, Kansas City, MO, USA). Finally, the dried fruit extract was kept at -80°C.

Determination of Chemical Constituents

The chemical constituents of the dried fruits of WKM, including moisture, crude proteins, fats, ash, and fibers were determined according to the standard methods of the AOAC (1997). The moisture and ash were determined using the weight difference method and the fiber content was estimated from the loss in weight of the crucible and its contents on ignition. The Kjeldahl method and Soxhlet extraction method were used for determination of the crude proteins and crude fat, respectively.

Determination of Phytochemical Compounds

Phytochemical screening was performed by determination of the total contents of the phenolics, flavonoids, and proanthocyanidin as they are the 3 most common groups of constituents providing natural antioxidant sources in plants (Lee *et al.*, 2004). Polyphenols, especially flavonoids, are highly effective scavengers of singlet oxygen and various free radicals and are reported to have several beneficial effects on human health (Baba and Malik, 2015, Montoro *et al.*, 2005). Proanthocyanidinins, a broad class of polyphenolic compounds polymerized by flavan-3-ol or flavan-3,4-diol (Jing *et al.*, 2015), are well recognized as the most effective antioxidants against free radicals (Lin and Zhang, 2002; Spranger *et al.*, 2008) and also have various strong biological effects (Jing *et al.*, 2015; Cadiz-Gurrea *et al.*, 2017).

Total Phenolic Content

The total phenolic content in the extracts was determined in triplicate using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Briefly, the extract or gallic acid (100 µL) and 2% sodium carbonate (2 mL) were added in a test tube. After 2 min, the Folin-Ciocalteu reagent (100 µL) was added, vortexed immediately,

and then allowed to stand at room temperature for 30 min. The absorbance of the extracts was measured spectrophotometrically at 750 nm. Gallic acid was used to prepare the standard curve. The concentration of the phenolic compounds of the extracts was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract.

Total Flavonoids Content

The total flavonoid content in the crude extracts was measured in triplicate using the aluminum chloride colorimetric method (Liu *et al.*, 2002). The extract or catechin (250 μ L), DI water (1.25 mL), and 5% sodium nitrite (75 μ L) were mixed together in a glass test tube. After 6 min, 10% aluminum chloride (150 μ L) was added and the mixture was allowed to stand at room temperature for 5 min. Finally 1M sodium hydroxide (500 μ L) was mixed with the solution. The absorbance of the extracts was measured spectrophotometrically at 510 nm. The flavonoid content of the extracts was expressed as milligrams of catechin equivalent (CE) per gram of dry extract.

Proanthocyanidins Content

A modified vanillin assay (Sun *et al.*, 1998) was adopted for quantification of the total proanthocyanidins content of the ethanolic plant extract in triplicate. An aliquot of 125 μ L of extract or catechin was added to a solution of 4% vanillin-methanol (750 μ L) and 4% HCl-methanol (375 μ L) in a glass test tube. The red coloration was read spectrophotometrically at 500 nm and catechin was used as the reference standard.

Determination of Antioxidant Properties

DPPH Assay

The antioxidant activity of the extracts was measured with respect to the hydrogen donating capability or radical scavenging activity towards the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The various concentrations of the extracts (0.2 mL) in ethanol were pipetted into a glass test tube and an ethanol solution of DPPH (3 mL) was added to each tube. The tube was shaken gently and left to stand in the dark at room temperature for

30 min. The absorption was measured at 517 nm by a spectrophotometer (Katsube *et al.*, 2004). The lower absorbance of the reaction mixture indicated higher free-radical scavenging activity. Inhibition of the free-radical DPPH in percentage was calculated as the following equation:

$$\text{Percent Inhibition} = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100 \quad (1)$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the reaction with the test compound. Trolox was used as a standard reference. The IC_{50} value, the concentration of the sample required for 50% scavenging of the DPPH free radical, was determined from the curve of percent scavenging plotted against the concentration. Each determination was carried out in quadruplicate.

FRAP Assay

The total antioxidant potential of the extract was determined using a ferric reducing ability of plasma (FRAP) assay (Benzie and Strain, 1996). The freshly prepared FRAP reagent contained 10 mM 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM HCl plus 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) and 0.3 M acetate buffer; pH 3.6 at the ratio of 1:1:10, respectively. Then various concentrations of the extracts (0.1 mL) were mixed with the FRAP reagent (3 mL). The absorbance was measured at 593 nm after standing for 30 min. The standard curve was prepared using different concentrations of the ascorbic acid standard solution. All determinations were performed in triplicate.

Dose Range-Finding Studies

The *in vitro* cytotoxicity of WKM on RAW264.7 was conducted in order to find the optimum dose-range for subsequent studies. Cells were incubated in the absence or presence of various concentrations of the WKM extract for 24 h. Cell viability was determined by the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and trypan blue exclusion methods.

One hundred μL of RAW264.7 cells were seeded in a 96-well plate at the concentration of 1×10^5 cells/well and were incubated at 37°C , 5% CO_2 overnight. The WKM extract was dissolved in DMSO to make a stock solution and diluted to various concentrations in media from 0.125 to 0.5 mg/mL (the final concentration of DMSO was 0.1%). After overnight incubation, the media were removed from the culture and were replaced with the extract or the vehicle control and further incubated for 24 h. Cell viability was determined with the MTT assay and trypan blue exclusion method.

MTT Method

After incubation with the WKM extract (0.125-0.5 mg/mL) for 24 h, the supernatant was removed. One hundred μL of 0.5 mg/mL MTT in PBS was added to each well, and cells were further incubated for another 4 h. The media were removed and formazan product was dissolved with 100 μL DMSO. The inhibition of cell growth induced by the WKM extract was determined using the optical density (OD) at 540 nm by a microplate reader.

The percentage of cell viability was calculated according to the following equation:

$$\text{Percentage of the viable cell} = \frac{\text{Average OD of test group}}{\text{Average OD of control group}} \times 100 \quad (2)$$

Trypan Blue Exclusion Method

After incubation of the WKM extract for 24 h, the supernatant was removed, the cells in each well were trypsinized by adding 20 μL of 0.25% trypsin-EDTA in PBS and then incubated at 37°C for 10 min. Then, the trypsin activity was neutralized by adding 100 μL media plus 10% FBS. The plate was placed on ice, 50 μL of cell suspension was pipetted into a micro centrifuge tube, and an equal volume of 0.4% (w/v) trypan blue was added and mixed thoroughly. A small amount of this mixer was loaded into a chamber of the haemocytometer. Then the viable (bright light) cells and dead (stained blue) cells were counted immediately under a light microscope at low magnification.

The percent viable cells were calculated according to the following equation:

$$\% \text{ Viable cells} = \frac{\text{Total viable cells per ml}}{\text{Total cells per ml}} \times 100 \quad (3)$$

Cellular Antioxidant Activity Assay

Intracellular antioxidant activity was evaluated using the DCFH-DA assay as described by Dufour *et al.* (2007). Briefly, RAW264.7 cells were plated in 96-well black clear-bottom plates at 1×10^6 cells/mL and incubated for 24 h at 37°C and 5% CO_2 . Cells were incubated either with various concentrations of the WKM extract (0.125, 0.25 and 0.5 mg/mL), or 10 μM quercetin. After 24 h, the cells were washed with Hank's balance salt solution (HBSS) and incubated for 30 min with HBSS containing 20 μM DCFH-DA. Cells were then washed again with HBSS 3 times and treated with 500 μM *tert*-butylhydroperoxide (*t*-BuOOH) in HBSS. Fluorescence was measured every 30 min and was followed for 3 h on the automated Gemini EM fluorescence microplate reader (Molecular Devices, San Jose, CA, USA) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Statistical Analysis

Homogeneous data were evaluated by using a 1-way analysis of variance (ANOVA), and differences between treatment groups and the vehicle control group were compared by using Tukey's HSD test. Non-homogenous data was evaluated by using a non-parametric analysis of variance. Significant differences were compared between the experimental groups and the vehicle control group. $P < 0.05$ was considered as statistically significant.

Results and Discussion

Proximate Composition of WKM

Proximate analysis of the dried ground fruits of the WKM demonstrated that crude protein was the main constituent ($71.44 \pm 2.25\%$) of the plant, followed by moisture ($11.96 \pm 1.39\%$), available carbohydrate ($10.88 \pm 1.17\%$), crude ash ($4.72 \pm 0.12\%$), crude fiber ($0.11 \pm 0.002\%$), and finally, crude fat ($0.9 \pm 0.08\%$) (Table 1).

The yield of the WKM crude extract was 12.65% of the dry weight.

Phytochemicals Composition of WKM

The total phenolic content of the WKM was analyzed by fitting the calibration curve of gallic acid ($R^2 = 0.997$) and expressed as mg gallic acid equivalent per gram of dry extract (mg GAE/g dry extract). The contents of the total flavonoids and proanthocyanidins were analyzed by fitting the calibration curve of catechin ($R^2 = 0.985$ and 0.951 , respectively) and were expressed as mg catechin equivalent per gram of dry extract (mg CE/g dry extract). The results in Table 2 showed that the total phenolic content of the extract was 56.75 ± 0.37 mg GAE/g dry extract, while the total flavonoid and proanthocyanidin contents were 5.03 ± 0.03 CE/g dry extract and 7.02 ± 0.12 mg CE/g dry extract, respectively. The phenolic content of the ethanolic extract of the WKM fruit is high in comparison with other fruits. The total phenolic content of goji berries (aqueous extract) was 14.13 ± 0.40 mg GAE/g dry extract (Benchennouf *et al.*, 2017), the ethanolic extracts of turkey berries (fruits of *Solanum torvum*) was 16.15 ± 0.23 mg GAE/g dry extract (Abdulkadir *et al.*, 2016), and of *Carissa carandas* was 17.10 ± 0.27 mg GAE/g dry extract (Mishra *et al.*,

2017). Concordantly, the total flavonoid content of the tested WKM was also higher than *C. carandas*. The flavonoid content of *C. carandas* was 2.69 ± 0.09 mg CE/g dry fruits which was almost 2 times less than the WKM. The proanthocyanidin content of the WKM was also higher than red and black rice grains which were found in the range of 0.58 ± 0.04 mg CE/g to 2.55 ± 0.14 mg CE/g.

Antioxidant Properties of Extract from WKM

Several methods have been developed and used to measure the total antioxidant activity of natural medicinal plants *in vitro*. Due to the complex composition of phytochemicals and oxidative process, the antioxidant capacity of medicinal plant extracts should not be investigated by using only 1 method (Bohm *et al.*, 1998). The use of at least 2 methods should be employed in order to evaluate the total antioxidant activity. Therefore, the present study investigates the antioxidant activities of the WKM extract by using both the DPPH and FRAP assays. Though the DPPH and FRAP assays are based on a single electron transfer reaction (Bunea *et al.*, 2011), their characteristics, sensitivities, mechanisms of the reaction, and endpoints are totally different. The DPPH

Table 1. Proximate composition of dried ground fruits of *Wan Khan Mak*

Proximate composition	WKM extract (%)
Moisture	11.96 ± 1.390
Crude protein	71.44 ± 2.250
Crude fat	0.9 ± 0.080
Ash	4.72 ± 0.120
Crude fiber	0.11 ± 0.002
NFE	10.88 ± 1.170

NFE = available carbohydrate. Data were mean $\pm$ SD., $n = 3$.

Table 2. Total phenolics, total flavonoids and proanthocyanidins contents of *Wan Khan Mak* fruit extract

Phytochemical component	WKM extract
Total phenolics (mg GAE/g dry extract)	56.75 ± 0.37
Total flavonoids (mg CE/g dry extract)	5.03 ± 0.03
Proanthocyanidins (mg CE/g dry extract)	7.02 ± 0.12

Data were mean $\pm$ SD., $n = 3$.

assay, 1 of the most frequently used methods to evaluate antioxidant activity, was employed to determine the ability of the samples to scavenge DPPH radicals, whereas the FRAP assay evaluates the antioxidant power of a sample by measuring its ferric reducing capability. The FRAP assays also evaluate the chain-breaking antioxidant potential (Ghiselli *et al.*, 1995; Benzie and Strain, 1996). The secondary metabolites in plants, vitamin C, Trolox, catechin, and quercetin were used as positive antioxidant controls in the present study as they are the most frequently used antioxidant standards for food samples.

The IC₅₀ values of the WKM extract in comparison with other commonly used antioxidants in the DPPH assay are shown in Table 3. The IC₅₀ of the WKM extract was 399.77±15.33 µg/mL, whereas the IC₅₀ of the antioxidant controls, quercetin, catechin, and Trolox were 2.002±0.022, 2.144±0.010, and 4.462±0.015 µg/mL, respectively. The result clearly showed that although the WKM possessed the capability to scavenge DPPH radicals, the extract was about 100-200 fold less efficient than quercetin, catechin, and Trolox. Notably, the scavenging ability of quercetin and catechin were comparable and were higher than Trolox.

In the FRAP assay, the antioxidant potential of the WKM extract was estimated from its ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) complex at low pH causing the formation of a colored ferrous-tripyridyltriazine complex (Gordon, 1990). The reducing properties are related to the ability of the compounds present in the extract to donate a hydrogen atom resulting in breaking the free

radical chain (Rice-Evans *et al.*, 1997; Irshad *et al.*, 2012). The reducing antioxidant power of the WKM extract in the FRAP assay was expressed as µg of ascorbic acid equivalent antioxidant capacity (AEAC) per gram dry weight of the extract and is shown in Table 2. The ferric reducing ability power or the FRAP value of the WKM extract was 44.07±0.51 µg AEAC /g dry wt.

It is well established that the major components of plant extracts that act as antioxidants are phytochemicals such as phenolic compounds, flavonoids, and proanthocyanidin. Saeed *et al.* (2012) found a correlation between the total phenolic and flavonoid contents in *Torilis leptophylla* with free radical scavenging capability. de Oliveira *et al.* (2012) reported a strong correlation between the total phenolic content and antioxidant activity of the crude extracts of *Sidastrum micranthum* and *Wissadula periplocifolia*. Irshad *et al.* (2012) also demonstrated that the antioxidant activity of *Cassia fistula* extracts was directly proportional to the contents of the phenolics and flavonoids. Jing *et al.* (2015) showed that the antioxidant activity and the scavenging capacity of hydroxyl radicals and DPPH• were increased in a concentration-dependent manner of proanthocyanidin in the extract of *Kunlun Chrysanthemum* flowers. The present study showed that phenolics, flavonoids, and proanthocyanidin were present in the WKM extracts when assessed with the Folin-Ciocalteu, aluminum trichloride, and vanillin methods, respectively. The WKM extracts also exhibited antioxidant activities when evaluated with the DPPH and FRAP assays. Notably, the

Table 3. DPPH free radical scavenging activity and ferric reducing ability power (FRAP) values of WKM extracts

Sample	Antioxidant Capacity	
	IC ₅₀ (DPPH) (µg/mL)	FRAP value (µg AEAC /g dry wt.)
WKM extract	399.77 ± 15.33	44.07 ± 0.51
Quercetin	2.002 ± 0.022	-
Catechin	2.144 ± 0.010	-
Trolox	4.462 ± 0.015	-

Data were mean ±SD, n = 3.

higher contents of phenolics and flavonoids in the WKM extracts also exhibited a higher antioxidant capacity in comparison with ethanolic mangosteen (*Garcinia mangostana* L.) hull extracts (Manasathien, 2017).

Therefore, the presence of phenolics, flavonoids, and proanthocyanidin in the WKM extracts could be associated to the scavenging activity of the DPPH radical and the ferric reducing power displayed in the FRAP assay.

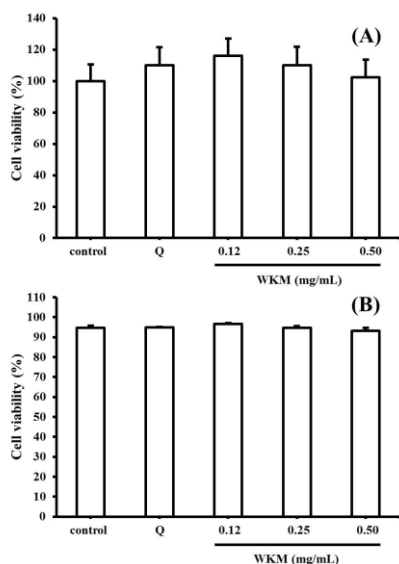


Figure 2. Cytotoxic effect of WKM extract and quercetin on cell viability of RAW264.7 cells as determined by MTT (A) and trypan blue exclusion (B)

Dose Range-Finding Studies

Prior to performing the cellular antioxidant study, a range finding test was performed to find the optimum concentration range of the WKM extract that was not cytotoxic towards RAW263.7. After the RAW264.7 cells were incubated with the VH control, various concentrations of the WKM extract (0.5, 0.25, and 0.125 mg/mL), or 10 μ M quercetin for 24 h, the cell viability was determined by the MTT assay and trypan blue exclusion method. The result in Figure 2 suggested that the WKM extract at 0.125–0.5 mg/mL had no cytotoxicity towards the

RAW264.7 cells, either determined by the MTT assay (Figure 2(a)) or trypan blue exclusion method (Figure 2(b)). No significant difference in cell viability was observed in the WKM exposed cultures when compared to the vehicle control group. The antioxidant positive control, 10 μ M quercetin, was also not cytotoxic. The cell viability of all groups was comparable and was more than 90%.

Although cell death is 1 of the most common consequences of the toxicity, some chemicals can exhibit a toxic effect by alteration of cellular metabolic activity without necessarily causing cell death. This aspect is called “cell vitality” (the physiological capabilities of cells) which represents a different aspect of cell functions from cell viability (the percentage of live cells in a whole population) (Kwolek-Mirek and Zadrag-Tecza, 2014). The MTT assay is based on the evaluation of the cell mitochondrial enzyme succinate hydrogenase activity in metabolizing MTT and hence the measurement of cell vitality rather than cell viability. Nevertheless, MTT is widely used for indirect determination of cell viability (Wang *et al.*, 2010). However, Maioli *et al.* (2009) reported that certain drugs and/or compounds could cause alterations in mitochondrial activity in the MTT assay. Actually, in the MTT assay, the cellular metabolic activity could be changed and variation of the results could be obtained by different conditions or chemical treatments including pH, green tea polyphenol (Plumb *et al.*, 1989; Hsu *et al.*, 2003), and color interference from media or samples. In the present study, the color interference from the sample and/or media in the MTT assay was minimized by subtracting the sample with a blank sample (extract plus media without cells). In addition, the MTT was prepared in colorless PBS, and the color interference from the extract was minimized by washing the WKM-incubated cells before the MTT addition.

Trypan blue exclusion is a conventional method for determination of cell viability. The method is based on the assumption that cell damage normally leads to cell death. At physiological pH, trypan blue dye is ionic and is excluded by the intact membrane of live

cells. Only dead or damage cells with an alteration in cell membrane integrity and permeability will allow the dye to go through and stain the nucleus and/ or cytoplasm. Therefore, only dead or damaged cells are stained blue (Strober, 2015).

In the present study, the determination of WKM toxicity towards RAW264.7 using both the cell viability and cell vitality approaches clearly indicated that the WKM extract at 0.125-0.5 mg/mL was not toxic to the RAW264.7 cells.

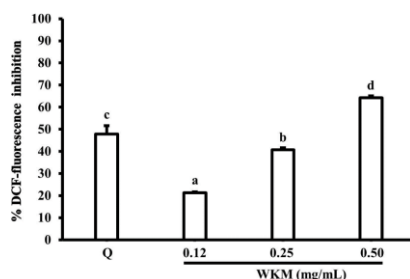


Figure 3. The percentage inhibition of DCF-fluorescence by WKM extract and quercetin in the DCFH-DA assay at 3 h. Q = 10 μM quercetin, WKM = Wan Khan Mak extract 0.125, 0.25 and 0.5 mg/mL. Means values with different letters are significantly different ($p < 0.05$, one-way analysis of variance)

Cellular Antioxidant Activity

Though both DPPH and FRAP are frequently used assays for antioxidant evaluation, they have some drawbacks for the measurement. In the DPPH assay, there is always a problem of color interference from other constituents in the crude extract, at the same wavelength of 515 nm. In addition, the DPPH radical is absent in living organisms. The drawbacks of the FRAP method are that the compounds with low redox potential (lower than 0.77) still can reduce the Fe^{+3} although they do not behave as antioxidants *in vivo*, some interfering compounds may absorb at the same wavelength, and the assay is also performed at a non-physiological pH (Pérez-Jiménez *et al.*, 2008). Therefore, the

antioxidant activity of the WKM extract was also evaluated by the cell-based assay, using DCF-DA as the intracellular fluorescent probe.

Cellular antioxidant activity assay is based on the ability of compounds to suppress the formation of DCF following *t*-BuOOH-mediated overoxidation of DCFH in the RAW264.7 cells. The increment of DCF fluorescence emission following reactive oxygen species (ROS)-mediated oxidation of DCFH was gradually increased after 30 min and reached a maximum level at about 3 h. Hence, the percentage of DCF scavenging by the WKM extract shown in Figure 3 was determined at 3 h. Preliminary study showed that after the 3 h period of *t*-BuOOH stimulation, the DCF fluorescent intensity gradually decreased (data not shown). The result clearly suggested that the WKM extract could significantly scavenge intracellular ROS induction in a dose-dependent manner. When compared to the vehicle control, the WKM extract at the concentrations of 0.125, 0.25, and 0.5 mg/mL inhibited ROS production in *t*-BuOOH-induced RAW264.7 cells by 21.24 ± 0.88%, 40.64 ± 0.86%, and 64.31 ± 1.59%, respectively, whereas the inhibition of 10 μM quercetin, the antioxidant positive control, was 47.84 ± 0.50%. Notably, the decrease of DCF formation was more pronounced in the highest concentration of the WKM (0.5 mg/mL) than the antioxidant positive control quercetin (10 μM).

Therefore, in addition to its extracellular antioxidant capacity, WKM also possessed a strong intracellular antioxidant activity in scavenging ROS and decreasing the oxidation of DCFH. Similarly, the crude water and ethanol extracts of various plants' leaves and fruits containing phenolic compounds had been shown previously to act as antioxidants in the cell-based assay using a DCFH-DA probe (Aiyegoro and Okoh, 2009; Shi *et al.*, 2011; Shukla *et al.*, 2012; Serra *et al.*, 2011). In addition, polyphenols are known to support bioactive activities in medicinal plants (Bunea *et al.*, 2011; Manasathien, 2017). Therefore, the antioxidant activity of WKM might be attributed to the presence of phenolics and flavonoids in the ethanol crude extract. Interestingly, although

WKM displayed lower DPPH radical scavenging activity than the antioxidant positive controls, quercetin, catechin, and Trolox (Table 2), the extract at 0.5 mg/mL could exhibit a higher potential to reduce ROS-induced oxidative stress in RAW264.7 cells than 10 μ M quercetin (Figure 3). This could be explained by differences in the modes of antioxidant action in both assays. The DPPH method is based on the scavenging activity of stable DPPH nitrogen radicals in the presence of antioxidants (Huang *et al.*, 2005). In a DCFH-DA assay, DCFH-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to form highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly (Yen *et al.*, 2003). Therefore, the observed antioxidant potential of the WKM extract in the DPPH and DCFH-DA assays could be mediated by a different mechanism and/or different antioxidant constituents in the extract. In fact, many important classes of antioxidants including flavonoids, 1 of the main chemical constituents in the WKM extract, contain a huge number of natural antioxidant compounds with a diversity of their modes of action (Kumar and Pandey, 2013). Depending on what specific phytochemical constituents present in the extract are providing the antioxidant activity, their discrete chemical structures, positions, numbers, and types of substitutions of features can influence their redox properties and hence their antioxidant potentials. The structure of antioxidant activity relationships of flavonoids have been studied; for example, multiple hydroxyl groups of the flavan nucleus of the flavonoid favor antioxidant and chelating activity, the methoxy group has unfavorable steric effects and an increase of lipophilicity, a double bond and carbonyl group in the heterocycle of the nuclear structure offer a more stable radical and hence increase the activity (Heim *et al.*, 2002). Phenolics may have hydroxyl or methoxyl groups; while flavones possess hydroxyl, keto, and free carboxylic groups and flavonoids have a flavan nucleus with different

types of substitutions. Typically, all of these compounds can provide different reducing properties. From the foregoing discussions, it is clear that not a single method can give a comprehensive prediction of antioxidant efficacy. Therefore, more than 1 method is recommended and there should be greater caution in extrapolating the extracellular data to intracellular-based assay (Aruoma, 2003).

Conclusions

Overall, the present results reveal that the WKM extract has no cytotoxicity towards RAW264.7 at the concentration range of 0.125-0.500 mg/ml and has potential antioxidant activities. However, further investigation in a long term toxicity study and more biological activities of the WKM extract are still required.

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