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Antioxidation and anti-melanogenesis of three colored flowers of *Dendrobium* hybrids

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ABSTRACT

Dendrobium is one of the major tropical orchid cut flowers for trading. The unqualified materials could possibly be used as sources of cosmetic ingredients. In this study, flowers of the *Dendrobium* hybrids, namely *D.* Sonia 'Jo Daeng' (purple), *D.* Khoa Sanan (white) and *D.* Black Pearl (purple black), were ethanolic extracted and determined for anti-melanogenesis activity. The optimum extracting condition based on antioxidant capacity using DPPH assay was sonicated, following with 100 rpm shaking at 50ºC for 6 h. While *D.* Black Pearl extract exhibited the highest total phenolic and flavonoid contents, *D.* Sonia 'Jo Daeng revealed the strongest DPPH scavenging activity. *D.* Khao Sanan extract had the highest metal chelating activity and anti-mushroom tyrosinase activity. All the extracts showed no cytotoxicity at the highest tested concentration (400 µg/mL). It was observed that *D.* Khao Sanan treated cells contained the lowest cellular melanin content. This implied that the metal chelating activity of the extracts exhibited some relationship to tyrosinase inhibition activity, which could be a useful screening assay for anti-melanogenesis agents. With relative effectiveness in melanogenesis inhibition to kojic acid, *D.* Khao Sanan orchid may be an applicable source of natural agent for skin lightening in cosmetic products.

Keywords: *Dendrobium* orchid; extracting condition; antioxidant; tyrosinase inhibition activity; anti-melanogenesis activity

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1. INTRODUCTION

Dendrobium is the second largest genus of the Orchidaceae family, mostly cultivated in Thailand. It is the main exported orchid at about 80% of total orchid cut flower exporters. The unqualified flower for trading could be left as waste. Alternative application of the waste is to make use of the cut flower as a valuable source for medical and cosmetic ingredients. *Dendrobium* has been widely used in traditional Chinese medicine as one of the fifty fundamental herbs to treat various ailments and as *Dendrobium* tonic for longevity (Xu et al. 2013; Lam et al. 2015). It has been claimed as a potential herb for age-related pathologic treatment such as anticancer, anti-diabetic, neuroprotective and immunomodulating activities. (Cakova et al. 2017). It also has cosmetic properties appropriate for skin dryness, skin wrinkle, and aging of skin (Kanlayavattanakul and Lourith 2020).

Various parts of orchid, their age or extracting condition could affect the attainable phytochemicals and their biological activity. 2,2-Diphenyl-1-picryl hydrazyl (DPPH) antioxidant activity and phytochemical content was primarily used to screen for the extracts (Obsuwan et al., 2019). Flowers of 1-

year-old *D.* Sonia 'Jo Daeng' provide higher phytochemical contents than those of the 4-year-old, as well as its antioxidant activity. Flower color also plays role on bioactive compounds and antioxidant activity. High levels of flavonoids and anthocyanin was reported in the purple or pink flower extract, while high polyphenol content was found in the white or yellow flower extract (Athipornchai and Jullapo, 2018; Nguyen et al., 2018). Those extracts from color flowers present high DPPH scavenging activity, white flower extract exhibits the highest ferrous iron-chelating abilities and yellow flower extract shows the highest ferric-reducing power (Nguyen et al., 2018).

The extracting conditions influence quality of the extract and its activity. The extract of *D. tosaense* under 50% ethanol at 50°C exhibits high total phenol content, antioxidant activity, and reducing power activity (Chan et al., 2018). While the extract under 50% ethanol at room temperature exhibits the strongest mushroom tyrosinase inhibition ability and the extracts using water either at 50°C or room temperature have strong effect similarly as kojic acid in melanin content inhibition under α-melanocyte-stimulating hormone (α-MSH) melanogenesis stimulation in B16F10 melanoma (B16F10) cells. This implied that obtainable information from in vitro assays may not give a same final result in living cells. As cutaneous pigmentation in human skin is under complex genetic control involving various cellular factors, rather than tyrosinase enzyme alone. Furthermore, antioxidant agent may inhibit melanogenesis by quenching reactive oxygen species (ROS), reducing cellular oxidative stress to lower the triggers of hormones or cytokines for melanogenesis stimulation. Therefore, this study aimed to find an optimal extracting condition of three flowers *Dendrobium* hybrids named *D.* Sonia 'Jo Daeng' (purple), Khoa Sanan (white) and Black Pearl (purple black), based on DPPH scavenging activity. The extract of each flower color was further analyzed for total phenolic content, total flavonoid content, DPPH assay, metal chelating assay, mushroom tyrosinase inhibition assay, and finally studied for melanin content assay in B16F10 cells.

2. MATERIALS AND METHODS

2.1 Materials

The reagents and chemicals used for phytochemicals and antioxidant activity assay including phosphate buffer saline (PBS), L-tyrosine and kojic acid were purchased from Sigma Chemical Co. (USA). The reagents for cell culture tests such as 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), Dulbecco minimum essential medium (DMEM), trypsin EDTA, penicillin-streptomycin were purchased from Gibco (USA). Fetal bovine serum (FBS) was purchased from Hyclone (USA). The flowers of *Dendrobium* hybrids including Sonia 'Jo Daeng', Khoa Sanan and Black Pearl were gifted from Mana Orchid Farm, Nakhon Pathom Province, Thailand. The samples were deposited at Sireeruckhachati Nature Learning Park, Mahidol University with voucher specimen number PBM-005643, PBM-005641 and PBM-005644, respectively.

2.2 Extraction method

The flowers were cleaned, air dried, milled to powder and kept at room temperature before use. The flower powder was extracted following the method of Chan et al. (2018) and Wang et al. (2018) with some modification. The extraction was done by maceration in 70% ethanol containing 0.02% hydrochloric acid using flower powder: solvent ratio at 1 g: 20 mL. They were extracted under 2 conditions, one was sonicated for 30 min using Vibra-cell TM ultrasonic (Sonics & Materials, Inc.,USA) (designated as S), and the other was non-sonicated (designated as NS). The extraction was done by shaking at 100 rpm at room temperature and 50°C, and different extraction periods at 2, 6 and 24 h. The samples at different concentrations (6.25-50 mg/mL) were then screened by DPPH assay to verify for the optimum extracting condition, which provided the highest scavenging activity. This was done 3 times. Then, the extracts under the selected condition were prepared in bulk for all tests. The solvent was removed to dryness under rotary evaporator, freeze dryer and kept in the 'As one' desiccator (Japan).

2.3 Determination of bioactive compounds

2.3.1 Total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu colorimetric method by Maisuthisakul et al. (2005) with slightly modification. Briefly, 30 µL of the extract (0.5, 1 and 2 mg/mL) and standard gallic acid solution (50-500 µg/mL) was mixed with 300 µL of 1 N Folin-Ciocalteu reagent, 150 µL of 15% Na_2CO_3 and 200 µL distilled water, using vortex mixer. A blank sample consisted of water and reagents was used as a reference. The resulting solution was kept in the dark at room temperature for 30 min, and the absorbance was read at 765 nm using LibraS22 Biochem spectrophotometer (Shimadzu, Japan). The phenolic content was computed and expressed as gallic acid equivalents (GAE mg/g).

2.3.2 Total flavonoid content (TFC)

TFC was measured by the method of Liu et al. (2002) with slight modification. Briefly, 250 µL of sample (1.5, 2.5 and 5 mg/mL) and catechin standard solution (25-300 µg/mL) was mixed with 75 µL of 5% $NaNO_2$ and 250 µL of 10% $AlCl_3$ $6H_2O$. The mixtures were incubated in the dark for 5 min and 500 µL of 1 M NaOH was added. The absorbance was measured after 15 min at 510 nm using a microplate reader (Biotek, USA). TFC was calculated using catechin as standard and expressed as catechin equivalents (CTE g/g).

2.4 Determination of DPPH radical scavenging activity

DPPH radical scavenging assay was measured by the method of Rattanachitthawat et al. (2010) and Sompong et al. (2011) with slight modification. The 20 µL of each extract at varying concentration (0.25-4 mg/mL) and ascorbic acid standard solution (12.5-100 µg/mL) was mixed with 180 µL of DPPH in ethanol. Deionized water was used as the control and the sample blanks were prepared by combining 20 µL of sample with 180 µL of ethanol. Absorbance was measured after 30 min incubation in the dark at room temperature. The percentage inhibition of the DPPH radical by the samples was calculated according to the Equation 1.

$$\% \text{ Radical scavenging inhibition} = \left[\text{A control} - \left(\frac{\text{A sample} - \text{A blank}}{\text{A control}}\right)\right] \times 100 \quad (1)$$

DPPH radical scavenging activity of sample was computed to the standard ascorbic acid and reported as mg vitamin C equivalent/g of sample.

2.5 Determination of metal chelating activity

The chelation of Fe^{2+} by the extracts was determined using Dinis' method (1994). Briefly, 12.5 µL of 10 mM $FeSO_4$ was added to 125 µL of sample solution at various concentrations (1-10 mg/mL). The reaction was initiated by the addition of 25 µL of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. Subsequently, the absorbance of the solution was measured at 562 nm. Sodium-EDTA was used as positive control at 10-80 µg/mL. The inhibition of ferrozine–Fe^{2+} complex formation in percentage was calculated as the Equation 2. Metal chelating activity of the samples were computed as mg EDTA equivalent/g of sample.

$$\% \text{ Inhibition} = \left[\text{A control} - \left(\frac{\text{A sample} - \text{A blank}}{\text{A control}}\right)\right] \times 100 \quad (2)$$

2.6 Determination of mushroom tyrosinase inhibitory activity

Tyrosinase inhibition of the extracts was measured using the method of Masuda et al. (2005) with some modification. L-Tyrosine was used as substrates whereas kojic acid was used as a standard tyrosinase inhibitor. The extracts were dissolved in 50% ethanol at a concentration of 3.0 mg/mL, and diluted to different concentrations using 50% ethanol. Then, 120 µL of 50 mM sodium phosphate buffer pH 6.8 was mixed with 20 µL of the extract solution and 20 µL of tyrosinase (800 unit/mL in 50 mM phosphate buffer, pH 6.8) in a 96-well plate. After incubated at room temperature for 10 min, 40 µL of 3.0 mM L-tyrosine substrate was added and the mixture was further incubated for 10 min. The absorbance of the reaction mixture was measured at 492 nm using microplate reader (Tecan, USA). The inhibition of tyrosinase activity was calculated as follows, and 50% inhibition (IC_{50}) was calculated.

$$\% \text{ Inhibition} = \left(\frac{\text{A blank} - \text{A sample}}{\text{A blank}}\right) \times 100 \quad (3)$$

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 test compound.

2.7 Determination of cytotoxicity in culture cells

B16F10 melanoma cells (ATCC® CRL 6475, USA) were cultivated in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells density at 1-3x10^4 cells/mL was seeded into each well of 96 well plates. The cultures were incubated overnight prior to addition of 25-400 µg/mL of the *Dendrobium* extract. After 72 h of incubation, cell viability was determined by MTT assay. Briefly, the MTT solution was added to the treated cells and the culture plate was incubated for 4 h. The formazan product in each well was dissolved by 50 µL dimethylsulfoxide (DMSO) and the absorbance was measured using microplate reader at 570 nm. The percent cell viability of each sample was calculated based on the cell control.

2.8 Determination of melanin content

The cellular melanin content was determined as previously described by Chen et al. (2016). In brief, B16F10 cells at 1-3x10^4 cells/mL were plated in 12-well plate and incubated for 24 h at 37°C. The extracts and kojic acid (positive control) at 300 µg/mL were separately added to the cell sheets, which were further incubated for 72 h. The treated cells were washed twice with PBS, and trypsinized for cell count. The same number of cells in the pellet from each treatment was dissolved in 100 µL of 1 N NaOH and 10% DMSO for 1 h in water bath at 80°C. The relative melanin content in percentage was computed from the absorbance read at 405 nm, comparing to that of the control cells.

2.9 Statistical analysis

Data were presented as mean ± standard deviation, computed from 3 experiments. The results from the cell cultures study were expressed as the mean ± standard error. Statistical analysis was performed using the SPSS program version 16.0 for Windows. The parameters were compared and analyzed using one sample t-test and ANOVA test with a significance level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Optimum extracting condition and the extracted yields

The antioxidant agent could also inhibit melanogenesis by quenching ROS, reducing cellular oxidative stress to lower the triggers of hormones or cytokines for melanogenesis stimulation. Thus, a rapid DPPH assay for antioxidant activity generally used for screening antioxidant agent was done to verify the optimum extracting condition in the present work. The IC_{50} of DPPH scavenging activity of each color flower was demonstrated in Table 1. The optimum extraction condition, which provided the lowest IC_{50} value and thus implied the highest DPPH scavenging activity for all flower extracts, was under 30 min sonication and following with 100 rpm shaking at 50°C for 6 h. The extraction condition of sonication and temperature at 50°C extraction might assist diffusion of phytochemicals from the flower tissue. Whereas, prolong extraction period could affect the quality of phytochemicals, similar to that reported by Wang et al (2018). The extraction of all samples under the optimum condition was then prepared in bulk for all experiments. The product yield after freeze drying of *D.* Sonia 'Jo Daeng', *D.* Khao Sanan and *D.* Black Pearl was 21.03±4.18, 20.12±2.55 and 20.8±2.26%, respectively.

Table 1. DPPH scavenging activity (IC_{50}) of Sonia 'Jo Daeng', Khoa Sanan and Black Pearl flower extracts under various conditions of extraction

Extracting condition		DPPH scavenging activity, IC_{50} (mg/mL)		
		Sonia 'Jo Daeng'	Khao Sanan	Black Pearl
RT 2h	NS	$5.45^{a} \pm 0.22$	$5.14^{a} \pm 0.56$	$5.13^{a} \pm 1.58$
	S	$4.25^{a} \pm 0.39$	$4.22^{a} \pm 1.10$	$3.82^{b} \pm 0.95$
RT 6h	NS	$5.59^{a} \pm 1.12$	$5.35^{a} \pm 1.09$	$5.12^{a} \pm 1.03$
	S	$4.32^{a} \pm 0.98$	$6.22^{b} \pm 1.20$	$3.82^{b} \pm 0.95$
RT 24h	NS	$9.20^{b} \pm 0.44$	$7.91^{b} \pm 1.35$	$5.56^{a} \pm 1.90$
	S	$12.51^{c} \pm 3.66$	$9.92^{c} \pm 2.03$	$5.20^{a} \pm 0.82$
50°C 2h	NS	$6.16^{a} \pm 0.87$	$6.77^{b} \pm 1.20$	$3.81^{b} \pm 0.57$
	S	$2.12^{d} \pm 0.14$	$1.98^{d,e} \pm 0.98$	$2.22^{c,d} \pm 0.80$
50°C 6h	NS	$3.02^{d} \pm 1.05$	$2.85^{d} \pm 1.05$	$2.83^{c} \pm 0.82$
	S	$1.24^{e} \pm 0.35$	$1.06^{e} \pm 0.38$	$1.73^{d} \pm 0.45$

Note: All values are mean ± standard deviation based on triplicate tests, S = 30 min sonication, NS = non-sonication, RT = room temperature, h = time of extraction period. $^{a, b, c, d, e}$ indicated significant difference among extracting condition of each flower (within column).

3.2 Phytochemicals and antioxidant activities of the flower extracts

Extract of each color flower was determined for the phytochemicals of TPC and TFC. Antioxidant activity based on DPPH scavenging assay and metal chelating assay were determined for antioxidant agent equivalent to vitamin C and EDTA content. The metal chelating activity of any agent could imply the activity of any protein. Heavy metals, such as Zn, Fe and Cu, are necessary for the suitable functioning of different proteins including tyrosinase. Therefore, metal chelating activity using Fe^{2+} was used to determine the antioxidant properties of compounds in this study. However, the activity on Cu^{2+} was also analyzed using mushroom tyrosinase inhibition assay.

TPC, TFC and antioxidant activity based on DPPH scavenging assay and metal chelating assay in the extract was shown in Figure 1. The extract from *D.* Black Pearl contained the highest TPC and TFC at 265.57±0.14 GAE mg/g and 25.34±0.34 CTE mg/g, respectively. It had been reported that purple flower orchid contained higher TFC than that of yellow and white orchid (Athipornchai and Jullapo, 2018; Nguyen et al., 2018). Additionally, much higher TPC (110.48-446.22 GAE mg/g) than TFC 8.10-38.70 QE mg/g had also been documented (Paudel et al., 2015; Nguyen et al., 2018). It could be observed that the extract of *D.* Sonia 'Jo Daeng' and *D.* Black Pearl exhibited higher DPPH scavenging activity than *D.* Khao Sanan. Alternatively, the extract of *D*. Khao Sanan provided the highest metal chelating activity at 8.37±0.86 mg/g equivalent to EDTA. This was similar to the finding that white and yellow flower extracts provided significantly higher iron-chelating rates to prevent the formation of ferrozine from ferrous ions than purple orchid (Nguyen et al., 2018). Since flavonoids could be an effective tyrosinase inhibitor (Lu et al., 2019), and metal chelating activity of the extract could chelate copper and inhibit tyrosinase activity (Kubo et al., 2000). Phytochemicals and antioxidant activities of the color flower extracts could, therefore, act as melanin inhibitor by different mechanisms.

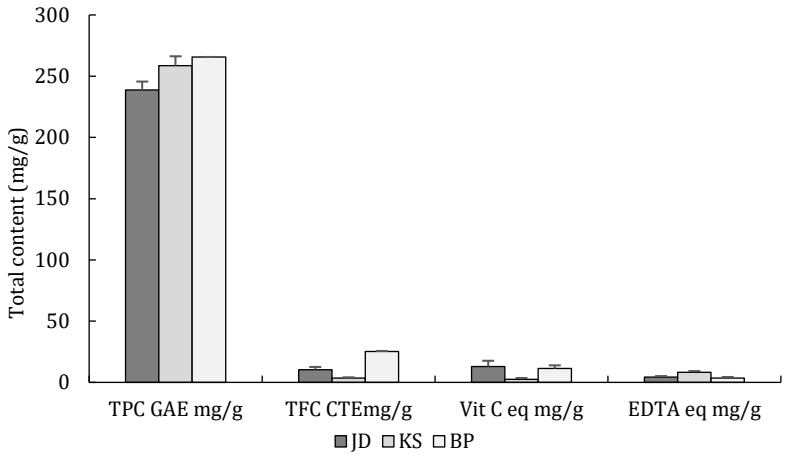

Figure 1. Total phenolic content and total flavonoid content, including antioxidant agent equivalent to vitamin C and EDTA in the extract sample of each color flower (JD=Sonia 'Jo Daeng', KS=Khoa Sanan, BP=Black Pearl)
Note: Vertical bars indicate the standard deviation (n=3), different letters indicate statistically significance among three sample ($p<0.05$).

3.3 Mushroom tyrosinase inhibition assay

Tyrosinase plays role on the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and on the oxidation of L-DOPA to dopaquinone, which are the initial steps in melanin biosynthesis. Therefore, tyrosinase inhibition assay is generally performed to verify for the

potential melanogenesis inhibitors. Kojic acid, used as control, exhibited the lowest IC_{50} and was the best tyrosinase inhibitor (Table 2). Interestingly, the extract of *D.* Khao Sanan had only 1.2 times lower tyrosinase inhibitory activity, compared to Kojic acid. While *D*. Sonia 'Jo Daeng' and *D.* Black Pearl provided 1.9 times and 3 times lower activity than kojic acid, respectively. Thus, *D.* Khao Sanan had the highest tyrosinase inhibitory activity among the tested flower extracts, which was well corresponded to the metal chelating activity. However, melanogenesis involves various cellular factors, therefore, the flower extracts were further analyzed using cell model for effective application.

Table 2. Inhibition concentration 50 (IC_{50}) of tyrosinase activity of each *Dendrobium* extracts and kojic acid

Samples	Tyrosinase inhibitory activity, IC_{50} (µg/mL)
Sonia 'Jo Daeng'	$340.27^{a} \pm 4.57$
Khao Sanan	$220.13^{b} \pm 1.54$
Black Pearl	$552.35^{c} \pm 3.79$
Kojic acid	$180.00^{d} \pm 0.45$

Note: All values are mean ± standard deviation based on triplicate tests.
$^{a, b, c, d}$ different letters indicate statistically significance at $p<0.01$

3.4 Cytotoxicity of the flower extracts

Cytotoxicity of the flower extracts was determined on B16F10 in the same condition that of the melanin content assay. It was revealed that all extracts were not cytotoxic to B16 F10 cells at all tested concentrations, ranging from 25 to 400 µg/mL (Table 3). Furthermore, treatment with the extracts at high concentration promoted cell growth and slightly activated melanin formation in the tested cells. This could be observed from few dendritic liked cells in the culture samples. Thus, B16F10 cells were likely differentiated to melanocyte as the cell concentration increased. Therefore, melanin content at the same cell density of treated samples were determined and computed with those under no treatment in melanin content assay.

Table 3. Percent cell viability of B16F10 after 72 h exposure to each color flower extract at various concentrations

Samples	% cell viability				
	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	400 µg/mL
Sonia 'Jo Daeng'	121.75±18.27	108.55±16.10	123.67±26.54	136.99±25.44	159.29±26.83
Khao Sanan	91.54±1.99	95.34±12.14	100.69±8.16	97.41±6.05	111.23±13.30
Black Pearl	101.47±11.53	110.87±17.35	117.13±16.68	132.23±8.97	153.41±16.99

Note: All values are mean ± standard deviation based on triplicate tests.

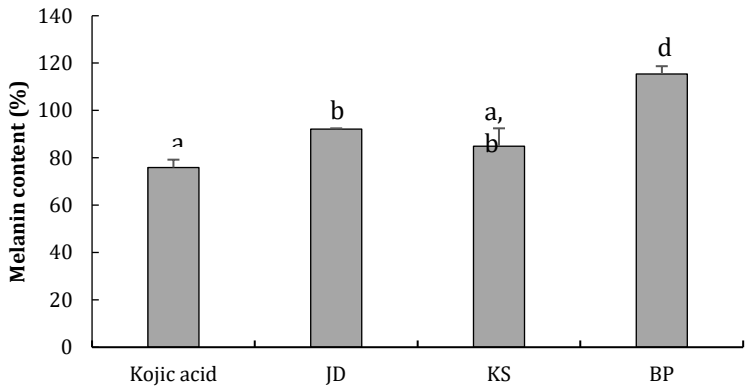

Figure 2. Melanin content of B16F10 treated with 300 µg/mL of each color flower extract of *Dendrobium sp.* for 72 h (JD=Sonia 'Jo Daeng', KS=Khoa Sanan, BP=Black Pearl)
Note: Vertical bars indicate the standard deviation (n =3-6), different letters indicate statistically significance at $p<0.05$

3.5 Melanin content assay

Melanin contents in B16F10 melanoma after treatment with flower extract or kojic acid at 300 µg/mL were presented in Figure 2. Kojic acid acts as melanin inhibitor by chelating Cu^{2+} in the active site of the enzyme tyrosinase (Cabanes et al., 1994). Interestingly, melanin content in *D.* Khao Sanan treated cells was comparable to that of kojic acid treatment. The mechanism of melanin inhibition could be by way of antioxidant activity and metal chelating activity of the phytochemicals in the extract. *D.* Khao Sanan extract could diminish melanin content at insignificant lower level than *D.* Sonia 'Jo Daeng' extract. However, significant lower melanin content in *D.* Khao Sanan treated cells was presented, as compared to that in *D.* Black Pearl treated cells. Thus, *D.* Khao Sanan extract was proven as a potential agent among all tested orchid extracts, which could be applicable for skin lightening or treatment in hyperpigmentation. *D.* Khao Sanan extract could also be superior to kojic acid, as kojic acid has been reported to have weak mutagenicity in bacteria by Ames test (Wei et

al., 1991). In addition, kojic acid has side effect by inducing allergic dermatitis (Nakagawa et al., 1995). Alternatively, all of the *Dendrobium* extracts were not cytotoxic in B16F10, and thus would be more applicable for safe use in cosmetic products.

4. CONCLUSION

The extracting condition and color of *Dendrobium* hybrids flower provided varying quantity of phytochemicals, antioxidant activity and melanogenesis inhibition properties. Sonication and 50°C extraction temperature were the optimum extracting method, which resulted in product with good antioxidant activity. Among all, *D.* Black Pearl extract exhibited the highest TPC and TFC. *D.* Sonia 'Jo Daeng' provided the highest DPPH scavenging activity while *D.* Khao Sanan extract revealed the highest metal chelating activity. The tyrosinase inhibitory activity and melanin content in B16F10 treated cells indicated that *D.* Khao Sanan extract was a promising material at relatively level with kojic acid for whitening skin care product.

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