PRINCE OF SONGKLA UNIVERSITY

Songklanakarin J. Sci. Technol.
42 (2), 383-390, Mar. - Apr. 2020

SJST
http://www.sjst.psu.ac.th

Original Article

Protective effects of lotus stamen extract on UV-B-irradiated skin cells activities

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Received: 11 September 2018; Revised: 4 December 2018; Accepted: 11 January 2019

Abstract

Traditionally, *Nelumbo nucifera* has been used as a tonic and stimulant. The aim of this study was to clarify a protective effect regarding UV-B induced inflammation and melanogenesis activities of the crude extract of the dried lotus stamen extract. The compounds of the extract were isolated by column chromatography. The anti-oxidant EC_{50} value of the crude extract was 0.0353 mg/mL while the EC_{50} values of the isolated compounds of kaempferol and kaempferol 3-O-β-D-glucopyranoside were 0.0030 and 0.0077 mg/mL, respectively, which were observed by DPPH assay. The tyrosinase inhibitory activity was tested by tyrosinase enzyme. The anti-tyrosinase IC_{50} value of the crude was 0.0192 mg/mL while the isolated kaempferol was 0.0496 mg/mL. To determine the anti-inflammatory activity, the molecular targets for the extract action were TNF-α and IL-6. The release of TNF-α and IL-6 was significantly reduced in UV-B-induced keratinocytes pretreated with the crude extract at concentrations from 25 to 100 μg/mL. Moreover, the crude extract was found to inhibit the melanin overproduction of B16F1 induced by α-MSH.

Keywords: lotus stamen, protective effect, melanogenesis inhibitory activity, anti-oxidant activity, anti-inflammatory activity

1. Introduction

Skin damage results from repeated exposure to external factors including ultraviolet (UV) rays, smoking, and poor dietary intake. However, skin damage is usually caused by overexposure to UV rays that consequently result in an accumulation of damaged macromolecules (Flament *et al.,* 2013; Viyoch *et al.,* 2012). Repeated exposure to UV, in particular UV-B, provokes the appearance of photodamaged skin such as mottled pigmentation and wrinkled and rough skin through various cellular mechanisms. For example, UV-B generates reactive oxygen species (ROS) which activate the

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release of pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) (Bashir, Sharma, & Werth, 2009; Tiraravesit *et al.,* 2015) from epidermal keratinocytes, thereby inducing overproduction of collagenases from dermal fibroblasts (Itsarasook, Ingkaninan, & Viyoch, 2014; Tiraravesit *et al.,* 2015). UV-B also activates the release of proopiomelanocortin, a precursor of α-melanocyte stimulating hormone (α-MSH), from keratinocytes (Chakraborty *et al.,* 1996; Corre *et al.,* 2004). This, in turn, results in the production and accumulation of melanin in the epidermal skin. α-MSH induces melanin production via activation of expression and activity of tyrosinase, a rate-limiting step enzyme of the melanogenesis process (Burchill, Ito, & Thody, 1993). A substance with multifunctional activities, that include anti-inflammatory and melanogenesis inhibitory activities, should be developed to prevent damage to the skin or improve photodamaged skin or both.

Nowadays, botanical extracts play an important role in health products because safe and effective compounds extracted from natural sources have been reported. Although an application has active ingredients, a purified substance is sometimes not necessary because purification may lead to the loss of synergistic effects of the botanical extract (Donsing, Limpeanchob, & Viyoch, 2008; Ebanks, Wickett, & Boissy, 2009; Tanaka, Misawa, Yamauchi, Abe, & Ishizaki, 2015). However, a standardized process to produce a reproducible extract and to quantify the pharmacological active compound(s) of an extract should be realized. Examples of extracts which are available in the market and have been used for health products are *Glycyrrihiza glabra* (licorice) extract, *Morus alba* L. (white mulberry) extract, and *Aloe vera* (aloe gel) extract.

Nelumbo nucifera Gaertn. (lotus) belongs to Nymphaeaceae family. This aquatic crop is found throughout the tropics. All parts of the lotus plant can be used for food and traditional medicine. In traditional Thai medicine, the stamen part of the lotus is used as a tonic and stimulant (Phonkot, Wangsomnuk, & Aromdee, 2008). Phytochemicals reported in the lotus stamen include phytosterol, kaempferol, and kaempferol derivatives (Paudel & Panth, 2015). Kaempferol is an anti-oxidant flavonoid reported to have several biological activities such as anti-oxidation, anti-inflammation, and anti-cancer activities (Chen & Chen, 2013; Taherkhani & Gheibi, 2014). However, the toxicity of kaempferol for topical application should be a concern because it was reported that it exhibited cytotoxicity to the human keratinocyte cell line and erythrocytes (Lee, Kang, Kim, Lee, & Cho, 2005; Vellosa *et al.,* 2011). Therefore, in the present study we aimed to observe the biological activities of lotus stamen crude extract as a photoprotective agent in topical products. The cytotoxicity of the crude extract was determined on human skin keratinocytes and melanocytes. Finally, we observed the effects of the crude extract to attenuate the release of pro-inflammatory cytokines, IL-6, and TNF-α from UV-B irradiated keratinocytes and to inhibit the production of melanin in α-MSH-induced melanoma cells. This is the first study to report the potential of the lotus stamen extract as an application to prevent photodamaged skin.

2. Materials and Methods

2.1 Materials

Lotus stamen was collected from Boraphet Lake, Nakhonsawan Province through a traditional Thai dispensary in Bangkok, Thailand. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3,4-Dihidroxyl-L-phenylalanine (L-DOPA), Melanocyte-stimulating hormone (α-MSH), tyrosinase from mushroom, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich, MO, USA. Kojic acid was purchased from Sigma-Aldrich, Steinheim, Germany. Dispase, amphotericin B, and fetal bovine serum (FBS) were purchased from Gibco, Auckland, New Zealand. Trypsin–EDTA solution was purchased from Gibco, Ontario, Canada. Keratinocyte-serum free medium (K-SFM), media 254, penicillin-streptomycin solution (10,000 units/mL penicillin and 10,000 μg/mL streptomycin) and supplements were purchased from Gibco, New York, USA). Sodium 3′-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) was purchased from Roche Diagnostics Corporation, Indiana, USA. Human tumor necrosis factor-α (TNF-α) and human interleukin-6 (IL-6) ELISA sets were purchased from eBioscience, CA, USA.

2.2 Preparation of lotus stamen extract

The air-dried stamen of *N. nucifera* were ground, extracted by soaking in MeOH at room temperature for 3 days, and then filtered. The process was repeated twice and the filtrates were then combined and evaporated under vacuum to dryness to give a brown residue.

2.3 Determination of active ingredients in lotus stamen

The crude MeOH extract was subjected to silica gel 60 column chromatography using mixtures of CH_2Cl_2-MeOH-H_2O as the mobile phase with gradient elution and was separated into 9 fractions (F1 to F9). Fractions F6 to F8 were selected for further isolation steps according to their relatively higher anti-oxidant activity. Column chromatography used a mixture of CH_2Cl_2-MeOH-H_2O as the eluent with gradient elution and Sephadex LH20 with MeOH as the eluent for compound isolation. The structures of the isolated compounds obtained from column chromatography were elucidated using nuclear magnetic resonance (NMR) spectroscopy (Bruker Ascend™ 400)

2.4 Determination of anti-oxidant activity

Anti-oxidative activities of the tested samples (crude methanol extract, fractions, and isolated compounds) were based on their free radical scavenging activity against DPPH, an organic radical model. The degree of DPPH decoloration indicated the scavenging efficiency of the added sample solution. This DPPH assay was performed in triplicate under a modified method (Donsing *et al.,* 2008; Viyoch, Mahingsa, & Ingkaninan, 2012). L-ascorbic acid and α-tocopherol were used as positive controls. The sample solutions of the tested

were prepared by dissolving in ethanol. The reaction mixture consisted of 4 mL DPPH (100 mM) and 200 μL of the sample solution. The sample solution was replaced with ethanol to serve as a blank solution. The mixture was mixed using a vortex mixer for 1 min and left to stand for 60 min at room temperature. After incubation, the absorbance of the remaining DPPH was measured at 517 nm wavelength. The radical scavenging activity was calculated as a percentage of DPPH decoloration. The EC_{50}, the equivalent concentration to give 50% effect, was determined by log-probit analysis using 6 to 10 different final concentrations of tested samples.

2.5 Determination of tyrosinase inhibitory activity

The tyrosinase inhibitory activities of the crude methanol extract and the isolated compounds were determined by measuring the content of dopachrome (Donsing *et al.,* 2008). The sample solutions of the extract, isolated compounds or positive control (kojic acid) were prepared by dissolving the crude extract or kojic acid in DMSO. An aliquot (20 μL) of the sample solution was mixed with 140 μL of 20 mM phosphate buffer (pH 6.8) and 20 μL of the aqueous solution of the mushroom tyrosinase (426 units/mL). This sample solution was replaced with phosphate buffer as a control. The resultant mixture was incubated at room temperature for 10 min, and 20 μL of 0.85 mM L-DOPA was then added to the mixture. After incubation at room temperature for 20 min, an amount of dopachrome was measured at the wavelength of 490 nm. The tyrosinase inhibitory activity of the tested sample was determined according to the decrease in production of dopachrome. The IC_{50}, the 50% inhibition of tyrosinase activity, was calculated by log-probit analysis using 6 to 10 different final concentrations of tested samples. This study was performed in triplicate.

2.6 Cytotoxicity of extract to human keratinocytes and melanocytes

2.6.1 Isolation of human skin keratinocytes and melanocytes

Cells were isolated from the epidermal layer of human excess surgery skin tissues (facial skin from women aged 50–60 years old). The protocol was approved by the Institutional Review Board of Naresuan University (project code: COA no. 53 02 04 0034). The skin tissues without the hypodermis layer were washed in phosphate buffered saline (PBS) containing antibiotics (100 units/mL of penicillin, 100 mg/mL of streptomycin, and 1 mg/mL of amphotericin B). After washing, the tissues were incubated overnight in 5% dispase solution at 4 °C to separate the epidermal from the dermal layer. The obtained epidermal was cut into small pieces and trypsinized in 0.25% trypsin–EDTA solution for 5 min at 37 °C in 5% CO_2. An equal volume of FBS was then added to the cell suspension to terminate the enzyme reaction of trypsin. The cell pellets were then harvested by centrifugation at 1,500 rpm for 5 min after trypsinization.

2.6.2 Cultivation of keratinocytes

The obtained cell pellets were resuspended in specific medium K-SFM with supplements (0.5 ng/mL epidermal growth factor 1-53 and 22 μg/mL bovine pituitary extract) and antibiotics. The cells were then incubated on type I collagen-coated plates at 37 °C in 5% CO_2 for 30 min. After incubation, non-adherent cells were removed, and the adherent keratinocytes were trypsinized and triturated to free them from their substrates. The keratinocytes were spun down at 1,500 rpm for 5 min after trypsinization. The collected cells were resuspended again in K-SFM with supplements and antibiotics, seeded at 1.7×10^6 cells/cm^3 in a 75 mL flask and grown at 37 °C in a humid atmosphere containing 5% CO_2. Early passages, with passage number not more than 2, were used in this study.

2.6.3 Cultivation of melanocytes

The obtained cell pellets were resuspended in specific medium, media 254 with supplements (20% FBS, 2% chelated FBS, 5 μg/mL L-glutamine, 15 μg/mL cholera toxin, 0.5 ng/mL basic fibroblast growth factor, 1.68 mM stem cell factor and 0.264 ng/ml endothelin-3) and antibiotics. The cells were seeded at a density of 2×10^5 cells/cm^3 in a 25 mL flask and grown at 37 °C in a humid atmosphere containing 5% CO_2. Passage number not more than 2 was used in this study.

2.6.4 Cell viability

The cells were transferred into each well (1×10^4 cells/well) of 96-well plates and cultured in specific medium containing supplements and antibiotics at 37 °C in a humid atmosphere containing 5% CO_2 for 24 h. After that, the cells were cultured in supplement-free and antibiotic-free medium containing various concentrations (5 to 100 μg/mL) of the extract. DMSO was used as a solubilizer and its final concentration did not exceed 0.1%. The highest concentration of the extract used was correlated to the limit of extract solubility in the final culture medium. After incubating the cells with the extract for 24 to 72 h, the medium in each well was replaced with 200 μL of fresh supplement-free and antibiotic-free medium. Subsequently, 50 μL of XTT solution was added. After the cells were further incubated for 4 h, the absorbance was read on a microplate reader at 490 nm, and the absorbance of the untreated cells was calculated as 100% viability. The study was performed in triplicate. The morphology of the cells was also observed under inverted microscope.

2.7 Determination of preventive effects of the extract on TNF-α and IL-6 released from UV-irradiated keratinocytes

UV radiation (275–305 nm, UV-B region) was emitted from a fluorescent sun lamp (FL8BLB, Toshiba Co., Tokyo, Japan) placed at 22 cm above the cell culture flasks. The UV intensity used was 70 J/cm^2. This intensity did not cause either cell death or alter the cell morphology, according to our previous studies (Tiraravesit *et al.,* 2015). Keratinocytes were transferred into each well (5×10^6 cells/well) of 6-well plates and cultured in K-SFM with supplements and antibiotics at 37 °C in a humid atmosphere containing 5% CO_2 for 48 h. After washing the cells in Ca^{2+}- & Mg^{2+}-free Hank's balanced salt solution (HBSS), the cells were cultured in supplement-free and antibiotic-free K-SFM containing various

concentrations (50–100 μg/mL) of the extract for 24 h. The cells were then washed and covered by Ca^{2+}- & Mg^{2+}-free HBSS before exposure to the UV irradiation. Following the UV exposure, the HBSS was replaced by supplement-free and antibiotic-free K-SFM for a further 4 h. After that, the cell-free supernatant was collected and kept at −80 °C until use for the analysis of TNF-α and IL-6 content by ELISA method. Viability of the UV-exposed cells was also observed by XTT assay according to the method described above. The study was performed in triplicate.

2.8 Determination of inhibitory effect of the extract on α-MSH-induced melanin production in mouse melanoma cell line (B16F1)

B16F1 cells were seeded into each well (9×10^5 cells/well) of 6-well plates and cultured in DMEM with 10% FBS and antibiotics at 37 °C in a humid atmosphere containing 5% CO_2 for 24 h. After cell-free supernatant was removed, the cells were washed with serum-free and antibiotics-free DMEM. The serum-free and antibiotic-free medium containing 1 nM α-MSH was added, and kojic acid at a concentration of 10 μg/mL (Donsing *et al.,* 2008) or extract at concentrations of 5–100 μg/mL was subsequently added. After treatment for 3 days, the treated cells were harvested for melanin content assay using the modified method (Buranajaree, Donsing, Jeenapongsa, & Viyoch, 2011; Donsing *et al.,* 2008) with a triplicate run. The harvested cells were washed with phosphate buffer saline, air-dried, and dissolved in 200 μL of 1 N NaOH containing 10% of DMSO. The resultant solutions were heated at 80 °C for 1 h and then cooled down at room temperature. The absorbance of melanin was measured at the wavelength of 490 nm. The melanin content was calculated by comparing to the absorbance of α-MSH-induced cells (without extract or kojic acid treatment) adjusted to 100%. A hemocytometer was used to count the viable cells that were not stained with blue dye.

2.9 Statistical analysis

All experimental data are expressed as mean±SD. Student's unpaired t-test was used to compare groups and $P<0.05$ was considered significant.

3. Results and Discussion

3.1 Anti-oxidants in the extract

The crude methanol extract of the air-dried stamen yielded 8.40% (w/w) of the dried stamen and provided anti-oxidant activity with an EC_{50} value of 0.0353 mg/mL. Anti-oxidative activity-guided quick chromatography of the crude methanol extract of *N. nucifera* stamen provided 9 fractions. The anti-oxidative activities (EC_{50}) of the first five fractions were more than 1 mg/mL while those of fractions F6, F7, F8, and F9 were 0.1191, 0.0684, 0.0731, and 0.2984 mg/mL, respectively. Further purification of fractions F6 and F7 was performed with column chromatography with CH_2Cl_2-MeOH-H_2O as the eluent and Sephadex LH20 with MeOH as the eluent. Structure elucidation of the isolated compounds was generated by NMR (Figure 1), sitosterol-3-O-β-D-glucopyranoside, and kaempferol, while kaempferol 3-O-β-D-glucopyranoside was found in fraction F8. The percent yields of sitosterol-3-O-β-D-glucopyranoside, kaempferol, and kaempferol 3-O-β-D-glucopyranoside were 0.0923%, 0.0072%, and 0.0083% (w/w) of the dried stamen, respectively. The isolated kaempferol and kaempferol 3-O-β-D-glucopyranoside (astragalin) provided anti-oxidant activity with EC_{50} values of 0.0068 and 0.0269 mg/mL, respectively, while the EC_{50} value of sitosterol-3-O-β-D-glucopyranoside could not be determined. The well-known anti-oxidants L-ascorbic acid and α-tocopherol provided EC_{50} values of 0.0030 and 0.0077 mg/mL, respectively. To clarify which compound(s) in the crude extract play a role in anti-oxidant activity, our study began with measurement of the hydrogen donating capacity of the crude methanol extract and the isolated compounds to the DPPH radical. This capacity is widely used to determine the anti-oxidant capacity of tested substances. The results demonstrated that kaempferol, which was found in the purified fractions of F6 and F7, presented the highest anti-oxidant activity. Moreover, the scavenging activity of kaempferol and its glucoside (astragalin) in this study was comparable to that in previous studies (Braca *et al.,* 2003; Liu *et al.,* 2013). Kaempferol is a plant flavonoid that was reported to have a wide range of biological activities including anti-oxidation, anti-inflammation, anti-tyrosinase, and anti-cancer activities (Chen & Chen, 2009; Kubo & Kinst-Hori, 1999; Taherkhani & Gheibi, 2014).

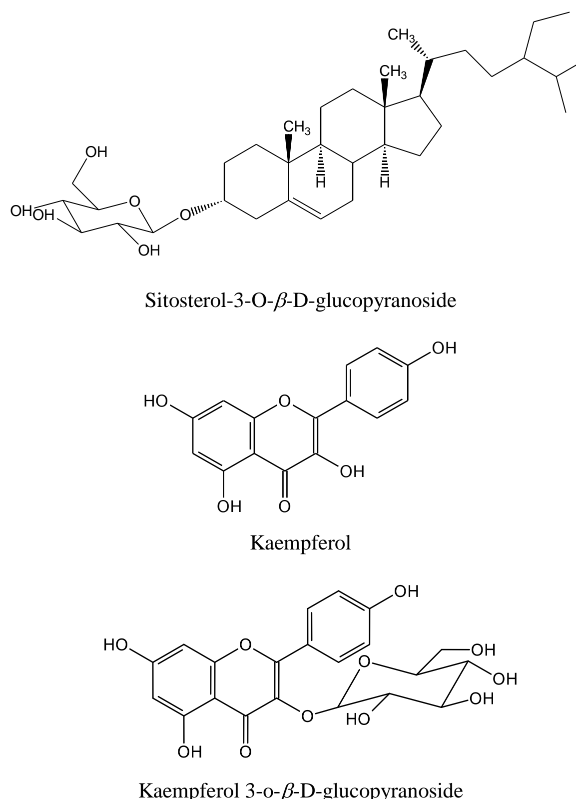

Figure 1. Chemical structure of compounds with anti-oxidant activity that were found in the lotus stamen crude extract isolated by column chromatography.

3.2 Tyrosinase inhibitory activity of the extract

The IC_{50} value of the crude methanol extract of lotus stamen was 0.0192 mg/mL while that of kojic acid, a well-known lightening agent was 0.0090 mg/mL. For the isolated compounds, sitosterol-3-O-β-D-glucopyranoside, kaempferol, and kaempferol 3-O-β-D-glucopyranoside (astragalin), we found tyrosinase inhibitory activity only in kaempferol with an IC_{50} value of 0.0496 mg/mL. The determination of tyrosinase inhibition activity found that the extract had lower tyrosinase inhibitory activity compared to kojic acid. However, compared to the reported tyrosinase inhibitory activity of *Morus alba* (mulberry) extract (IC_{50}, 0.0783 mg/mL) (Wang *et al.,* 2006), another well-known natural depigmenting agent, the lotus stamen methanol extract seems to be a stronger tyrosinase inhibitor. This indicated the potential use of the lotus stamen extract to prevent and/or improve skin hyperpigmentation, which is one of the major symptoms of photodamaged skin. For the isolated compounds, our findings indicated that kaempferol plays a role in tyrosinase inhibitory activity of the lotus stamen methanol extract. Generally, tyrosinase is a copper-containing monooxygenase enzyme involved in two steps of melanin synthesis: 1) hydroxylation of tyrosine to 3, 4 dihydroxyphenylalanine (DOPA, monophenolase activity) and 2) the oxidation of DOPA to dopaquinone (diphenolase activity). In this study, the inhibitory activity was concerned with diphenolase inhibitory activity of mushroom tyrosinase since L-DOPA was used as the substrate. Previous studies (Kubo & Kinst-Hori, 1999; Taherkhani & Gheibi, 2014) reported that kaempferol could inhibit diphenolase activity of mushroom tyrosinase and such inhibition activity presumably resulted from chelating copper in the enzyme. Moreover, kaempferol is a flavonoid that has hydroxyl groups, which structurally relates to DOPA. For this reason, another possible mechanism is that kaempferol is a competitive inhibitor of tyrosinase.

3.3 Cytotoxicity to human skin keratinocytes and melanocytes

The action of the extract in the determination of cytotoxicity was tested with concentrations of 5–100 μg/mL and incubation times of 24–72 h. The extract did not show any effect on the viability of the treated cells (Figures 2A and 2B). Additionally, the morphology of the cells treated with the extract did not change. Therefore, this concentration range was selected for further studies in pro-inflammatory cytokine release and melanin synthesis. Keratinocytes are the skin cells that are initially exposed to external stimuli. Therefore, keratinocytes were used in this study to test the cytotoxicity of the lotus stamen methanol extract. Since human melanocytes are a target of the extract action, they were also used to test the cytotoxicity of the extract. The extract did not show any effects on the viability and morphology of the treated cells. Cytotoxicity of kaempferol or its derivatives or both to human keratinocyte cell line, HaCaT, and erythrocytes was reported (Lee *et al.,* 2005; Vellosa *et al.,* 2011). However, in the present study, cytotoxicity of the crude extract that consisted of kaempferol to human keratinocytes and melanocytes was not found. The crude extract possibly contains some substances that protected the cells from damage in response to external stimuli.

3.4 Preventive effects of the extract on TNF-α and IL-6 released from UV-irradiated keratinocytes

The level of TNF-α and IL-6 released by human keratinocytes is shown in Figure 3. In the non-UV-B irradiation state, the release of TNF-α and IL-6 into the culture medium was not detectable according to the sensitivity of ELISA assays used. However, the keratinocytes were activated to release large amounts of TNF-α and IL-6 after UV-B irradiation for 4 h. TNF-α and IL-6 releases were significantly reduced in cells pretreated with the extract (at concentrations that ranged from 25 to 100 μg/mL (39.87, 39.16, 24.74 and 23.53%, respectively) for TNF-α, 75 to 100 μg/mL (42.22 and 40.07%, respectively) for IL-6). The reduced releases were not caused from the reduction of the viable cells (data not shown). UV-B radiation not only induces free radical generation, but also induces inflammation through changes in the production of cytokines by keratinocytes and other skin cells (Tiraravesit *et al.,* 2015; Yoshimizu *et al.,* 2008). Changes in the levels of free radicals and cytokines are the trigger of several phenomena involved in UV-induced skin damage or aging or both. An accelerated degradation of collagen in dermal skin mediated by cytokine-induced collagenase expression pathway and generation of immunosuppression mediated by suppressor T cells are examples of such phenomena

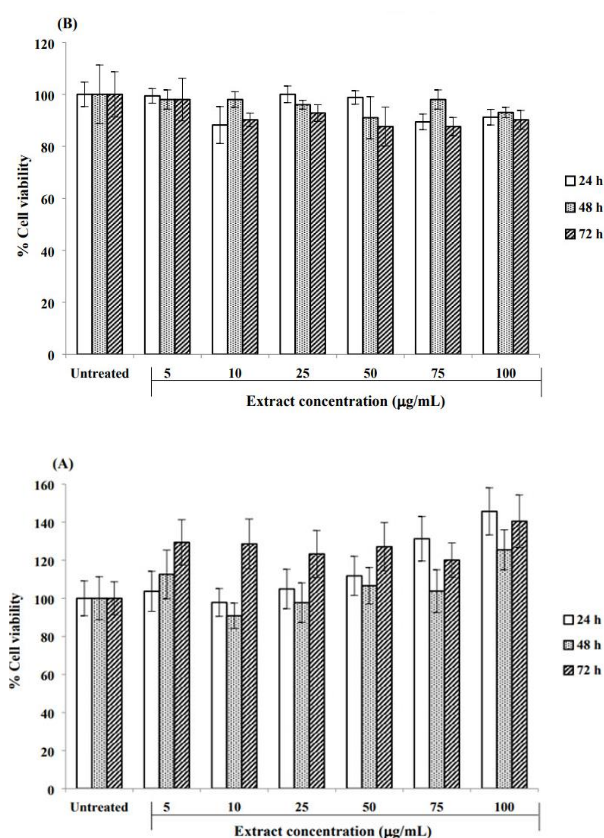

Figure 2. Effect of the crude extract of lotus stamen (5 to 100 μg/mL) on viability of human skin keratinocytes (A) and melanocytes (B). Data are expressed as percent of untreated ells, and each column represents mean±SD of a triplicate study.

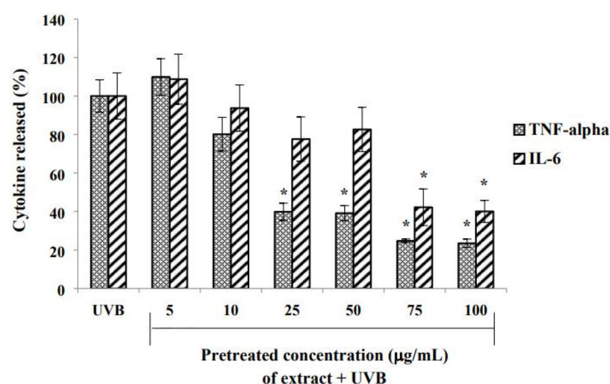

Figure 3. Effect of the crude extract of lotus stamen on TNF-α and IL-6 released from UV-B-irradiated human skin keratinocytes. Cells were pretreated with the extract (5 to 100 µg/mL) for 24 h before irradiation with UV-B (70 J/cm^2). Cell-free supernatants were collected to detect TNF-α and IL-6 using ELISA. Data are expressed as mean±SD of a triplicate study.
*P<0.05 compared with UV-B-irradiated cells without pretreatment with the extract (Student's t-test).

(Poon, Barnetson, & Halliday, 2005; Schwarz, 2005; Tiraravesit *et al.,* 2015; Yoshimizu *et al.,* 2008). As a result of UV-accelerated inflammation and skin disorders, we are interested in observing the effects of the extract on the production of TNF-α and IL-6. These pro-inflammatory cytokines are usually released from keratinocytes after UV exposure to human skin and influence the release of other cytokines involved in skin damage and aging (Yoshimizu *et al.,* 2008). According to our findings, the levels of TNF-α and IL-6 released by human keratinocytes correlated with previous studies which indicated up-regulation of these cytokines in UV-B-irradiated keratinocytes or skin or both (Bashir *et al.,* 2009; Grandjean-Laquerriere, Le Naour, Gangloff, & Geunounou, 2003; Tiraravesit *et al.,* 2015). Basically, free radicals including ROS can provoke nuclear factor κB (NFκB) (Bashir *et al.,* 2009), a transcription factor corresponding to cytokine expression. Therefore, the reduced cytokine release was possibly attributed to impeding ROS activity or ROS downstream signaling or both. Moreover, recent studies reported the anti-inflammatory activity of kaempferol, a plant flavonoid via suppressing expression and activity of NFκB and activator protein-1 (Kang *et al.,* 2008; Kim *et al.,* 2015). Indeed, TNF-α and IL-6 have shown an inhibitory effect on melanin synthesis (Cichorek, Wachulska, Stasiewicz, & Tymińska, 2013; Englaro *et al.,* 1999). Since UV irradiation enhances melanin production in skin through several factors, including α-melanocyte stimulating hormone (α-MSH), this conflicts with up-regulation of TNF-α and IL-6 after UV exposure. A previous study suggested that up-regulation of TNF-α might be part of a negative feedback system to prevent hyperpigmentation (Englaro *et al.,* 1999). Nevertheless, skin continually exposed to UV cannot preserve the balance of this system thereby leading to skin hyperpigmentation along with photo-damage. Therefore, application of a substance that has the ability to decrease the overproduction of cytokines would provide a beneficial use in preventing skin damage.

3.5 Inhibitory effect of the extract on α-MSH-induced melanin production in mouse melanoma cell line (B16F1)

In this study, we evaluated the potential effect of the extract on prevention of overproduction of melanin in α-MSH-induced B16F1 melanoma cells (Figure 4). The extract was found to inhibit the melanin overproduction of B16F1 in a dose-dependent manner. Under similar test conditions, kojic

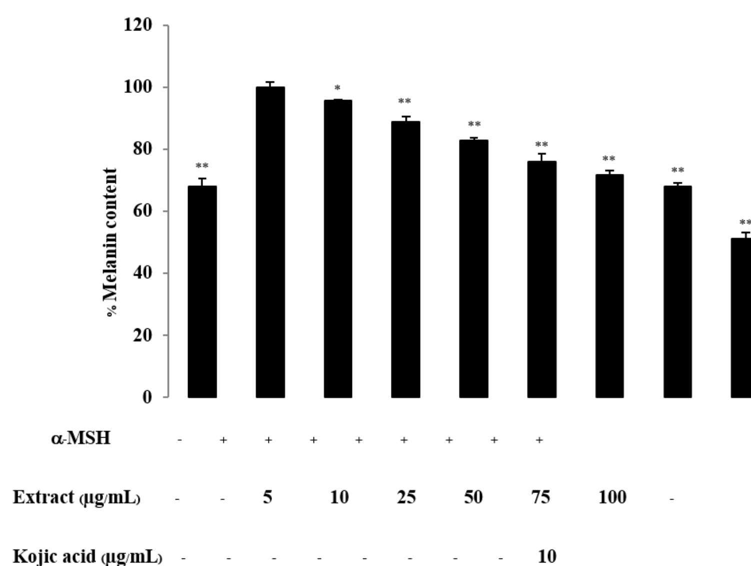

Figure 4. Effect of kojic acid (10 µg/mL) and the crude extract of lotus stamen (5 to 100 µg/mL) on melanin production from α-MSH-induced B16F1 cells. Cells were treated with 1 nM α-MSH followed by kojic acid or the extract. Melanin was extracted from the collected cells and the absorbance measured at 490 nm. Data are expressed as mean±SD of a triplicate study.
*P<0.05 and **P<0.01 compared with α-MSH-induced B16F1 cells without treatment with the extract or kojic acid (Student's t-test).

acid at a concentration of 10 μg/mL also inhibited increasing melanin in α-MSH-induced B16F1 (51%). The number of viable cells after treatment with the extract did not significantly change compared with the untreated cells (data not shown). Pigmentation or melanin synthesis is a biochemical process that plays a role in the protection of skin from UV radiation. However, overproduction of melanin indicates skin disorders. These disorder appearances include freckles, senile lentigines, and melasma. As mentioned above, chronic exposure to UV causes hyperpigmentation because UV radiation induces secretion of several factors including α-MSH. The secreted α-MSH from keratinocytes stimulates tyrosinase production and activity in melanocytes. Our study indicated that the extract could suppress the melanin production without affecting cell viability. At a similar concentration (10 μg/mL), kojic acid showed a stronger melanin reduction than the extract. However, at higher concentrations, the potential of melanin reduction of the methanol extract was close to kojic acid. The reduction of melanin production in α-MSH-induced melanoma cells was likely caused from tyrosinase inhibitory activity of kaempferol in the extract, according to the results from the study in tyrosinase inhibitory activity. Kaemferol was reported to have depigmenting activity by direct or indirect tyrosinase inhibition (Kubo & Kinst-Hori, 1999; Taherkhani & Gheibi, 2014). Nevertheless, skin pigmentation is comprised of two steps: 1) melanin synthesis within melanosomes and 2) the distribution of melanosomes to keratinocytes. The other inhibitory mechanisms such as melanosome distribution of the extract should be a concern and further clarified.

4. Conclusions

In this study, we focused on the protective effect of a crude methanol extract of dried lotus stamen regarding UV-B-induced inflammation and melanogenesis activities that to our knowledge has never been reported. The extract demonstrated its anti-inflammatory (protective effect) activity via reduction of TNF-α and IL-6 releases by UV-B-irradiated keratinocytes. Moreover, the study in α-MSH-induced B16F1 indicated that the crude extract could reduce overproduction of melanin. Sitosterol-3-O-β-D-glucopyranoside, kaempferol, and kaempferol 3-O-β-D-glucopyranoside were found to be anti-oxidants contained in the crude extract, and kaempferol showed the highest free radical scavenging activity and tyrosinase inhibitory activity. We hypothesized that kaempferol of the crude extract plays a dominant role in anti-melanogenesis activities and has a protective effect regarding UV-B-induced inflammation. With all findings taken together, the crude methanol extract of the dried lotus stamen showed the potential for application as a photopreventive agent. However, further studies on the safety and efficacy of the extract should be conducted at the clinical level to determine its beneficial use as a dermo-pharmaceutical agent.

Acknowledgements

We thank the grant from Naresuan University for the financial support of the research and the International Laboratories Corp. Ltd., Thailand for providing the lotus stamen extract. We also thank the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Thailand for the use of their facilities.

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