Evaluation of lotus stamen extract on anti-inflammatory and melanogenesis inhibitory activities

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Original Article

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

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Abstract

Traditionally, *Nelumbo nucifera* has been used as 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’ extract were isolated by column chromatography. The anti-oxidant $EC_{50}$ value of the crude was 0.0353 mg/mL while that of the isolated compounds, kaempferol and kaempferol 3-O-$\beta$-D-glucopyranoside was 0.0030 and 0.0077 mg/mL, respectively which was 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 whilst the isolated kaempferol was 0.0496 mg/mL. For determining anti-inflammatory activity, the molecular targets for the extract action were TNF-$\alpha$ and IL-6. The releasing of TNF-$\alpha$ and IL-6 was significantly reduced in UVB-induced keratinocytes pretreated with the crude extract at concentration of 25 to 100 $\mu$g/mL. Moreover, the crude extract was found to inhibit the melanin overproduction of B16F1 induced by $\alpha$-MSH.

Keywords: Lotus stamen; protective effect; melanogenesis inhibitory activity; anti-oxidant activity; anti-inflammatory activity

1. Introduction

Skin damage is resulted from repeated exposure to external factors including ultraviolet (UV) ray, smoking, and poor dietary intakes. However, skin damage is majorly caused by overexposure to UV ray that consequently results in an accumulation of damage macromolecules (Flament et al., 2013; Viyoch et al., 2012). Repeated exposure to UV, in particular UVB, provokes the appearance of photodamaged skin such as mottled pigmentation, and wrinkled and rough skin through various cellular mechanisms. For
examples, UVB generates ROS activating the release of pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) (Tiraravesit et al., 2015; Bashir Sharma, & Werth, 2009) from epidermal keratinocytes, thereby inducing overproduction of collagenases from dermal fibroblasts (Tiraravesit et al., 2015; Itsarasook, Ingkaninan, & Viyoch, 2014). The UVB also activates the release of proopiomelanocortin, a precursor of α-melanocyte stimulating hormone (α-MSH), from keratinocytes (Corre et al., 2004; Chakraborty et al., 1996). This, in turn, reflects 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 melanogenesis process (Burchill, Ito, & Thody, 1993). To maximize capacity of prevention and/or improvement of photodamaged skin, therefore, application of substance with multifunctional activities including anti-inflammatory and melanogenesis inhibitory activity should be concerned.

Nowadays, the botanical extract plays an important role in health products. This is because safe and effective compounds extracted from the natural sources have been reported. As application as an active ingredient, a using of purified substance is sometimes not necessary because such purification may lead to lose the synergistic effects of the botanical extract (Tanaka, Misawa, Yamauchi, Abe, & Ishizaki, 2015; Ebanks, Wickett, & Boissy, 2009; Donsing, Limpeanchob, & Viyoch, 2008). However, a standardized process to produce a reproducible extract and to quantify a pharmacological active compound(s) of extract should be realized. Examples of the extracts which are available in the market and have been used for health products are *Glycyrrhiza 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 tropical. Every parts of lotus can be used for food and traditional medicine. For traditional Thai Medicine, the stamen part of lotus has been used as tonic and stimulant (Phonkot, Wangsomnuk, & Aromdee, 2008). Phytochemicals reported in the lotus stamen includes phytosterol, kaempferol and kaempferol derivatives (Paudel & Panth, 2015). Kaempferol is an anti-oxidant flavonoid that has been reported for several biological activities such as anti-oxidation, anti-inflammation and anti-cancer (Taherkhani & Gheibi, 2014; Chen & Chen, 2013). However, the toxicity of kaempferol for topical application should be concerned as there has been report about its cytotoxicity to human keratinocyte cell line and erythrocytes (Vellosa et al., 2011; Lee, Kang, Kim, Lee, & Cho, 2005). In the present study, therefore, we are interested to observe the biological activities of lotus stamen crude extract for acting as photoprotective agent in topical product. The cytotoxicity of the crude extract was determined on human skin keratinocytes and melanocytes. Finally, the effects of the crude extract to attenuate the release of pro-inflammatory cytokines, IL-6 and TNF-α, from UVB-irradiated keratinocytes and to inhibit the production of melanin in α-MSH-induced melanoma cells were observed. This is the first time to report the potential of the lotus stamen extract for application in prevention of photodamaged skin.

2. Materials and Methods

2.1 Materials

Lotus stamen was collected from Boraphet Lake, Nakhonsawan Province through a Thai traditional dispensary in Bangkok, Thailand. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 3, 4-Dihydroxy-L-phenylalanine (L-DOPA), Melanocyte-stimulating hormone (α-MSH), tyrosinase from mushroom and dimethylsulfoxide
(DMSO) were purchased from Sigma-Aldrich, Missouri, USA. Kojic acid was purchased from Sigma-Aldrich, Steinheim, Germany. Dispase, amphotericin B and fetal bovine serum (FBS) was 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, California, 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$_2$Cl$_2$-MeOH- H$_2$O 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 step, according to their relatively higher anti-oxidant activity. A column chromatography using a mixture of CH$_2$Cl$_2$-MeOH-H$_2$O as eluent with gradient elution and Sephadex LH20 using MeOH as eluent was used as a technique for compound isolation. The isolated compounds obtained from column chromatography were elucidated their structure by using nuclear magnetic resonance (NMR) spectroscopy (*Bruker* AscendTM 400)
2.4 Determination of anti-oxidant activity

Anti-oxidative activity of the tested samples (crude methanol extract, fractions and isolated compounds) is 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 with triplicate under modified method (Viyoch, Mahingsa, & Ingkaninan, 2012; Donsing et al., 2008), and L-ascorbic acid and α-tocopherol were used as a positive control. The sample solutions of the tested were prepared by dissolving with ethanol. The reaction mixture consisted of 4 mL DPPH (100 mM) and 200 μL of the sample solution. This sample solution was replaced with ethanol for acting as a blank solution. The mixture was mixed using vortex for 1 min and left to stand for 60 min at room temperature. After incubation, the absorbance of remaining DPPH was measured at wavelength of 517 nm. The radical scavenging activity was calculated as a percentage of DPPH decoloration. EC\textsubscript{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 activity of the crude methanol extract and the isolated compounds was determined by measuring 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 for acting 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 produced was measured at wavelength of 490 nm. The tyrosinase inhibitory activity of the tested sample was determined according to the decrease in production of dopachrome. 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 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 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 in 5% dispase solution for overnight at 4°C to separate epidermal from 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 in cell suspension to terminate enzyme reaction of trypsin. The cell pellets could be 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. Cells were then incubated onto type I collagen-coated plate at 37°C in 5% CO₂ for 30 min. After incubation, non-adherent cells were removed, and the adherent keratinocytes were trypsinized and triturated to free 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 x 10^6 cells/cm² in 75-cm² flask and grown at 37°C in a humid atmosphere containing 5% CO₂. 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 density of 2 x 10^5 cells/cm² in 25-cm² flask and grown at 37°C in a humid atmosphere containing 5% CO₂. Passage number not more than 2 was used in this study.

2.6.4 Cell viability

The cells were transferred into each well (1 x 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% CO₂ for 24 h. After that, cells were cultured in supplements-free and antibiotics-free medium containing various concentrations (5 to 100 µg/mL) of the extract. DMSO was used as a solubilizer and its final concentration was not exceeded 0.1%. The highest concentration of the extract used was correlated to
the limit of extract solubility in the final culture medium. After incubating cells with the
electrodes for 24 to 72 h, the medium in each well was replaced with 200 μL of fresh
supplements-free and antibiotics-free medium. Subsequently, 50 μL of XTT solution was
added. After cells were further incubated for 4 h, the absorbance was read on a microplate
reader at 490 nm, and the absorbance of the untreated cell was calculated as 100%
viability. The study was performed in triplicate. Morphology of 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

A UV radiation (275 – 305 nm, UVB 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/cm². This selected intensity did not cause cell death
and/or alter cell morphology, according to our previous studies (Tiraravesit et al., 2015).
Keratinocytes were transferred into each well (5 x 10⁶ cells/well) of 6-well plates and
cultured in K-SFM with supplements and antibiotics at 37°C in a humid atmosphere
containing 5% CO₂ for 48 h. After washing cells in Ca²⁺& Mg²⁺-free Hank’s balanced
salt solution (HBSS), the cells were cultured in supplements-free and antibiotics-free K-
SFM containing various concentrations (50 to 100 μg/ml) of the extract for 24 h. The cells
were then washed and covered by Ca²⁺& Mg²⁺-free HBSS before exposing to UV
irradiation. Follow the UV exposure, the HBSS was replaced by supplements-free and
antibiotics-free K-SFM for further 4 h. After that, cell-free supernatant was collected and
kept at -80°C until used for analysis of TNF-α and IL-6 content by ELISA method.
Viability of the UV-exposed cells was also observed by XXT 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 x 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% CO₂ for 24 h. After cell-free supernatant was removed, the cells were washed with serum-free and antibiotics-free DMEM. The serum-free and antibiotics-free medium containing 1 nM α-MSH [18] was added, and kojic acid at concentration of 10 μg/mL (Donsing et al., 2008) or the extract at concentration of 5 to 100 μg/mL was subsequently added. After treatment for 3 days, the treated cells were harvested for melanin content assay by using the modified method (Donsing et al., 2008; Buranajaree, Donsing, Jeenapongsa, & Viyoch, 2011) with 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 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%. Hemocytometer was used for counting viable cell that not being stained with blue dye.

2.9 Statistical analysis

All experimental data are expressed as mean ± S.D. 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 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 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 with column chromatography with CH$_2$Cl$_2$-MeOH-H$_2$O as eluent and Sephadex LH20 with MeOH as eluent, and structure elucidation of the isolated compounds (Figure 1) by NMR, sitosterol-3-O-β-D-glucopyranoside and kaempferol were found, while kaempferol 3-O-β-D-glucopyranoside was found in fraction F8. Percent yield of sitosterol-3-O-β-D-glucopyranoside, kaempferol and kaempferol 3-O-β-D-glucopyranoside was 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$_{50}$ of 0.0068 and 0.0269 mg/mL, respectively while 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$_{50}$ of 0.0030 and 0.0077 mg/mL, respectively. To clarify that which compound(s) in the crude extract play a role in anti-oxidant activity, our study began with measurement of hydrogen donating capacity of the crude methanol extract and the isolated compounds to DPPH radical. This capacity is widely used to determine anti-oxidant capacity of the tested substance. The resulted demonstrated that kaempferol which found in purification of fractions 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 (Liu et al., 2013; Braca et al., 2003). Kaempferol is a plant flavonoid, that has been reported a wide range of biological activities including anti-oxidation, anti-inflammation, anti-tyrosinase and anti-cancer (Taherkhani & Gheibi, 2014; Chen & Chen, 2009; Kubo & Kinst-Hori, 1999).

3.2 Tyrosinase inhibitory activity of the extract

The IC\textsubscript{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-\beta-D-glucopyranoside, kaempferol and kaempferol 3-O-\beta-D-glucopyranoside (astragalin), we found tyrosinase inhibitory activity only in kaempferol with IC\textsubscript{50} of 0.0496 mg/mL. The determination of tyrosinase inhibition activity found that the extract has lower tyrosinase inhibitory activity when compared to kojic acid. However, as comparison to a reported tyrosinase inhibitory activity of Morus alba (mulberry) extract (IC\textsubscript{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 indicates a potential use of the lotus stamen extract for prevention and/or improving skin hyperpigmentation, one of the major symptoms of photodamaged skin. For the isolated compounds, our finding indicates 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. The previous studies (Taherkhani & Gheibi, 2014; Kubo & Kinst-Hori, 1999) have 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 having hydroxyl groups, which structurally relates to DOPA. In 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 extract in the determination of cytotoxicity was tested with the concentrations of 5 to 100 µg/mL and incubation time (24 to 72 h), the extract did not show effect on the viability of the treated cells (Figure 2A and 2B). Additionally, the morphology of cells treated with the extract was not changed. This concentration range, therefore, was selected for further studies in pro-inflammatory cytokine release and melanin synthesis. Concerning on cytotoxicity to human skin, keratinocyte is kind of skin cell firstly exposed to external stimuli. Cytotoxicity of the lotus stamen methanol extract to this cell was, therefore, tested in this study. Moreover, the cytotoxicity of the extract to human melanocytes, a target of the extract action was tested. The extract did not show effect on the viability and morphology of the treated cells. There has been reported about cytotoxicity of kaempferol and/or its derivatives to human keratinocyte cell line, HaCaT, and erythrocyte (Vellosa et al., 2011; Lee et al., 2005). However, in the present study, the cytotoxicity to human keratinocytes and melanocytes of the crude extract consisting of kaempferol was not found. The crude extract possibly contains some substances that are protecting the cell 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. At non-UVB irradiation state, TNF-α and IL-6 released into the culture medium was not detectable, according to sensitivity of ELISA assays used. However, keratinocytes was activated to release large amounts of TNF-α and IL-6 after UVB irradiation for 4 h. TNF-α and IL-6 releases were significantly reduced in cells pretreated with the extract (at concentration in range of 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 reduction of releases did not cause from the reduction of the viable cells (data not shown). UVB radiation can not only induce free radicals generation, but also induced inflammation through changes in production of cytokines by keratinocytes and other skin cells (Tiraravesit et al., 2015; Yoshimizu et al., 2008). The changes in levels of free radicals and cytokines are the trigger of several phenomena involving in UV-induced skin damage and/or aging. An accelerated degradation of collagen in dermal skin mediated by cytokines-induced collagenase expression pathway and a generation of immunosuppression mediated by suppressor T cells are examples of such phenomena (Tiraravesit et al., 2015; Yoshimizu et al., 2008; Poon, Barnetson, & Halliday, 2005; Schwarz, 2005). As a result of UV-accelerated inflammation and skin disorders, we are interested in observing the effects of the extract on production of TNF-α and IL-6. These pro-inflammatory cytokines are majorly released from keratinocytes after UV exposure to human skin and influence on release of other cytokines involving in skin damage and aging (Yoshimizu et al., 2008). For the level of TNF-α and IL-6 released by human
keratinocytes, our finding correlates to the previous studies indicating up-regulation of these cytokines in UVB-irradiated keratinocytes and/or skin (Tiraravesit et al., 2015; Bashir et al., 2009; Grandjean-Laquerriere, Le Naour, Gangloff, & Geunounou, 2003). Basically, free radicals including ROS can provoke nuclear factor κB (NFκB) (Bashir et al., 2009), a transcription factor corresponding to cytokine expression. The reduction of cytokine releases was, therefore, possibly attributed to impeding ROS activity and/or ROS downstream signaling. Moreover, recent study has reported anti-inflammatory activity of kaempferol, a plant flavonoid via suppressing expression and activity of NFκB and activator protein-1 (Kim et al., 2015; Kang et al., 2008). Indeed, TNF-α and IL-6 have shown inhibitory effect on melanin synthesis (Englaro et al., 1999; Cichorek, Wachulska, Stasiewicz, & Tymińska, 2013). As 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. Previous study suggested that up-regulation of TNF-α might be a part of a negative feedback system for preventing 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 the substance that shows ability to decrease 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 as shown in Figure
4. The extract was found to inhibit the melanin overproduction of B16F1 in a dose-dependent manner. Under similar test condition, kojic acid at concentration of 10 \( \mu \)g/mL also inhibited increasing melanin in \( \alpha \)-MSH-induced B16F1 (51%). Number of viable cells after treating with the extract was not significantly changed, as comparing to the untreated cells (data not shown). Pigmentation or melanin synthesis is a biochemical process that plays a role in protection 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 as UV radiation induces secretion of several factors including \( \alpha \)-MSH. The secreted \( \alpha \)-MSH from keratinocytes stimulates tyrosinase production and activity in melanocytes. Our study indicates that the extract could suppress the melanin production without affecting cell viability. At a similar concentration (10 \( \mu \)g/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 closed to that of kojic acid. The reduction of melanin production in \( \alpha \)-MSH-induced melanoma cells might predominantly causes from tyrosinase inhibitory activity of kaempferol in the extract, according to the results from the study in tyrosinase inhibitory activity. Kaemferol has been reported on depigmenting activity by direct or indirect tyrosinase inhibition (Taherkhani & Gheibi, 2014; Kubo & Kinst-Hori, 1999). 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 concerned and further clarified.

4. Conclusion
In this study, we emphasize a protective effect of the crude methanol extract of the dried lotus stamen regarding UV-B induced inflammation and melanogenesis activities that has never been reported. The extract was clarified its anti-inflammatory (protective effect) activity via reduction of TNF-α and IL-6 releases by UVB-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 highest free radical scavenging activity and tyrosinase inhibitory activity. We hypothesized that kaempferol predominantly plays a role in a protective effect regarding UV-B induced inflammation and melanogenesis activities of the crude extract. With all findings taken together, the crude methanol extract of the dried lotus stamen showed the potential for application as protopreventive substance. However, further studies on safety and efficacy of the extract should be conducted in clinical level to determine its beneficial use as dermo-pharmaceutical agent.

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Antioxidant and free radical scavenging activity of flavonol glycosided from different

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Figure 1. Chemical structure of compounds with anti-oxidant activity, which were found in the lotus stamen crude extract isolated by column chromatography.
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 cells, and each column represents mean ± S.D. of triplicate study.
Figure 3. Effect of the crude extract of lotus stamen on TNF-α and IL-6 released from UVB-irradiated human skin keratinocytes. Cells were pretreated with the extract (5 to 100 μg/mL) for 24 h before irradiation with UVB (70 J/cm²). Cell-free supernatant were collected to detect TNF-α and IL-6 by using ELISA. Data are expressed as mean ± S.D. of triplicate study. *p < 0.05, when compared with UVB-irradiated cells without pretreatment with the extract (Student’s t-test).
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 1 nM α-MSH followed by kojic acid or the extract. Melanin was extracted from the collected cells and measured the absorbance at 490 nm. Data are expressed as mean ± S.D. of triplicate study. *p < 0.05 and **p < 0.01, when compared with α-MSH-induced B16F1 cells without treated with the extract or kojic acid (Student’s *t*-test).
Figure 1 Specify the caption of the figure.

Figure 2 Specify the caption of the figure.