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Inhibition of two stages of melanin synthesis by sesamol, sesamin and sesamolin

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ABSTRACT

Objective: To investigate the antimelanogenesis properties of three sesame compounds-sesamol, sesamin and sesamolin via two stages of melanin synthesis vis-à-vis sunscreen function and enzyme inhibition in melanoma cell line in order to search for alternative depigmenting agents.

Methods: Antimelanogenic effects of sesame lignans were assessed in SK-MEL2 compared with the reference depigmenting agents, kojic acid and β-arbutin, in order to evaluate: (a) the sunscreen function of sesamol, sesamin and sesamolin by measurement of UV absorbtion property; (b) the inhibition of tyrosinase activity through mushroom and cellular tyrosinase; and (c) the effect on melanin content and melanogenic protein expression (tyrosinase, TRP-1 and TRP-2) by Western blot analysis; and (d) the toxicity of sesamol, sesamin and sesamolin to cells using cell cytotoxicity assay.

Results: The results showed that sesamin, sesamolin and sesamol exerted satisfiable sunscreen function by absorbed UVB at 290 nm. Sesamol exhibited the highest inhibition of mushroom tyrosinase activity, but lipophilic sesamolin exhibited the highest cellular tyrosinase inhibition (IC_{50} of 1.6 μM) followed by sesamin, sesamol, and kojic acid, respectively. The order from high to low inhibition of melanin pigment was detected in the SK-MEL2 treated with sesamolin, sesamin, sesamol, kojic acid, and β-arbutin, respectively. Sesamolin and sesamin successfully inhibited cellular tyrosinase activity and respectively decreased TRP-1/TRP-2 (36%/15%) and TRP-1 levels (16%), thereby inhibiting melanogenesis via antityrosinase activity. No cytotoxicity to SK-MEL2 or Vero (normal) cell lines was observed at the lignan concentrations that exerted an anti-melanogenic effect.

Conclusions: Three sesame lignans prevent melanin synthesis through 2 stages: (a) by blocking melanin-induction and (b) by interrupting melanogenic enzyme production. This study provides evidence that sesamol, sesamin and sesamolin are potential for anti-melanogenesis agents.

1. Introduction

Melanocytes are important cells, responding to skin phenotype and hair pigmentation. Melanin is the product of melanocytes-special dendritic cells of neural crest origin that migrates to the skin. Melanocytes contribute to skin tone and provide direct protection against the damage caused by ultraviolet (UV) radiation. Exposure to UV radiation-a powerful carcinogen (95% of UVA and 5% of UVB)-will lead melanocytes to melanogenesis, also known as tanning [1,2]. Melanogenesis depends on an interaction between the keratinocytes and melanocytes. The events occur during two-way communication, in which keratinocytes induce melanocytes to synthesize melanin, after which it transfers into the keratinocytes [3].

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The regulation of pro-opiomelanocortin by p53 in human cells occurs when epidermal keratinocytes are exposed to UV radiation, which induces cellular and DNA damage. This activation of the pro-opiomelanocortin gene leads to generation of α-melanocyte-stimulating hormone, which binds to melanocortin 1 receptor on melanocytes and activates a signaling cascade-viz., (1) the cyclic adenosine monophosphate (cAMP) and (2) protein kinase A pathways. The cAMP signaling pathway induces generation of cAMP via G proteins coupled with the melanocortin 1 receptor. G proteins activate adenylate cyclase in melanocytes, thereby converting adenosine triphosphate into cAMP. cAMP triggers activation of the protein kinase A signaling pathway followed by upregulation of the cAMP response element-binding protein transcription factors, which promote transcription of microphthalmia-associated transcription factor. Subsequently, microphthalmia-associated transcription factor increases up-regulation transcription of pigment enzymes [tyrosinase, tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2)], resulting in melanin production [4].

Melanin is synthesized either as a dark color, brown-black insoluble eumelanin or a light color, red-yellow alkali soluble, sulfur-containing phaeomelanin. Eumelanin has two proteins involved in melanin biosynthesis-viz., TRP-1 and TRP-2. Melanin biosynthesis begins from the hydroxylation of tyrosine by monophenolase action and the oxidation of 3, 4-dihydroxyphenylalanine (L-DOPA) to *o*-dopaquinone by diphenolase. The rate-limiting step, melanogenesis, occurs during the oxidation of tyrosine by tyrosinase [5]. When dopachrome is produced by tyrosinase, it may be enzymatically transformed into 5,6-dihydroxyindole-2-carboxylic acid by TRP-2. 5,6-dihydroxyindole-2-carboxylic acid is further oxidized to form indole-5,6-quinone carboxylic acid by TRP-1. Finally, these dihydroxyindoles are oxidized to eumelanin. Increasing melanin biosynthesis can darken the skin and induce a number of skin hyperpigmentations (*i.e.*, freckles, melasma and solar lentigo) [6].

Available whitening agents (*i.e.*, hydroquinone) have been reported to cause undesirable effects including the induction of skin cancer [7]. Natural whitening compounds (*e.g.*, kojic acid) are also reported to induce cytotoxicity, skin cancer and dermatitis. In some countries, this agent has been banned for cosmetic use [8], so there is a market for safe and effective skin whitening agents, which remain popular in Asia Pacific, and Latin America. A number of efforts have been made to develop new effective agents against pigmentation abnormalities, using novel biologically-active compounds from plants and other natural sources [9]. The trends in the market of consumer preference for sun care products are rising with multi-functional whitening sunscreen and sunscreen with herbal medicines and natural ingredients. The global market for sun care products was postulated to be approximately USD 11.1 billion by 2020 [10]. It is more challenging for research and development of sun protection products with advanced and novel ingredients.

Sesame seeds (*Sesamum indicum* L.) and oil contain important bioactive compounds lignans, which play an important role in health-promoting effects [11]. Sesame ingestion (50 g sesame seed powder daily for 5 weeks) in post-menopausal woman has positively affected sex hormones (up and down), increased antioxidant status and decreased blood lipids [12]. The sesame lignans, (sesamin and sesamolin) and sesamol are reported to have many pharmacological properties: antioxidant activity [13], enhancing antioxidant activity of vitamin E in lipid peroxidation systems [14]; antiproliferative activity [15]; lowering cholesterol levels [16]; antihypertensive effects [17,18]; increasing hepatic fatty acid oxidation enzymes [19]; neuroprotective effects against hypoxia or brain damage [20]; and finally, antitumor, anti-mutagenic, anti-carcinogenic [21], and anti-inflammatory properties [22].

Moreover, sesamol has been reported to affect melanogenesis related to inhibition of tyrosinase [23]. The antimelanogenic properties of sesamin, sesamol and sesamolin have not yet been thoroughly assessed. The effect of sesame compounds (sesamin, sesamol and sesamolin) on melanogenesis was investigated using SK-MEL2 human melanoma cells. Their effect on sunscreen function and the melanogenic protein expression of tyrosinase, TRP-1 and TRP-2 that are involved with catalytic intermediates, in the chain of melanin biosynthesis were examined. This is the first report of the functions of sesame compounds on sunscreen function and inhibition of melanogenic protein expression.

2. Materials and methods

Sesamol was purchased from spectrum chemical (USA). Sesamin and sesamolin used in this study were the gift form Associate Professor Dr. Kwanjai Kanokmedhakul. Sesamin and sesamolin were isolated from sesame, purified, and structures elucidated by using Nuclear magnetic resonance. Dimethyl sulfoxide was from Sigma (France). Mushroom tyrosinase, β-arbutin and neutral red were purchased from Sigma–Aldrich Chemical Co. (USA). Kojic acid was from TCI (Japan). L-DOPA was from Acros Organic Geel (Belgium). Tyrosinase antibody was from Abcam (UK). TRP-1, TRP-2, β-actin and goat anti-mouse IgG_1-horseradish peroxidase were purchased from Santa Cruz Biotechnology (USA). The BCA protein assay kit and the Spectra™ multicolor broad range protein ladder were purchased from Thermo Scientific (USA) and (Lithuania), respectively. The protease inhibitor cocktail was from Amresco (USA). The Amersham™ ECL™ prime blotting detection reagent was purchased from GE Healthcare (UK). The DMEM medium, fetal bovine serum and penicillin/streptomycin were purchased from GIBCO (USA). The microplate reader was from Anthos 2010 (AnthosLabtec Instruments, Ges.m.b.H, Austria).

2.1. Determination of sunscreen function

Ultra violet radiation is contributing to the skin tone. The blocking or less exposure to ultra violet radiation will lead to skin lightening. UV absorbing property determined by using spectral scanning function The blocking assay was tested following Sapkota *et al.* [24] based on the assumption that the compound which absorbs UVA 320–400 nm or UVB 290–320 nm ranges will have sunscreen effect. Sesamol, sesamolin, sesamin and the positive control (*viz.*, kojic acid and β-arbutin) were dissolved in methanol with a final concentration of 3.33 mM. The optical density was scanned from 200 nm to 400 nm using UV–visible spectrophotometer [24].

2.2. Determination of tyrosinase inhibition activity

Tyrosinase is a key enzyme in melanin biosynthesis. The inhibition of tyrosinase enzyme activity leads to skin whitening;

hence, the inhibition of mushroom tyrosinase activity *in vitro* was performed primarily according to the method of Momtaz *et al.* [25] with minor modifications. Mushroom tyrosinase enzyme was added to each of the wells of a 96-well plate for a final concentration of 27 units/mL. Test compounds-sesamin, sesamol sesamolin and positive control (kojic acid and β-arbutin) were added to each well. The final concentrations of sesamin, sesamol and sesamolin were in the range of (10–200) μg/mL, (0.1–0.5) μg/mL, and (20–100) μg/mL, respectively. And the final concentrations of positive controls were (1–10) μg/mL for kojic acid and (200–800) μg/mL for β-arbutin. The prepared substrate L-DOPA solution in 0.1 M PBS pH 6.8 was added to the reaction mixture yielding a final concentration of 4.5 mM. The reaction mixture was incubated at room temperature (26 °C) for 20 min and measured at 492 nm using a microplate reader. The concentration possessing 50% tyrosinase inhibition-compared to the control (in an absence of the test compound) or IC_{50} value was calculated using the following equation:

$$\%\text{Inhibition} = \frac{(\text{Absorbance}_{\text{without test compound}} - \text{Absorbance}_{\text{with test compound}}) \times 100}{\text{Absorbance}_{\text{without test compound}}}$$

2.3. Cell culture

The African green monkey kidney cell line (Vero) (ATCC# CCL-81) and the melanoma cell line (SK-MEL2) (CLS-Cell Lines Service, Germany) were grown in DMEM medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Cell cytotoxicity assay

To determine the safety of sesame lignans and the positive controls in cell model, the cytotoxicity assay was performed *in vitro* in both normal (Vero) and SK-MEL2 cell lines. Cytotoxicity was evaluated using neutral red (NR) assay [26,27]. Viable cells accumulate and bind neutral red within the lysosomes, but dead cells cannot because of the fragility of the lysosomal membrane and irreversible molecular alterations in the dead cells [28]. SK-MEL2 cells or Vero cells (5×10^4 cells per well) in complete DMEM medium-were added to a 96-well plate and incubated at 37 °C in 5% CO_2 for 24 h. Afterward the cells were treated with sesame compounds of for 48 h. The final concentrations used for sesamin and sesamolin were 10, 25, 50 μg/mL, while final concentration of sesamol were 200, 400, 600 and 800 μg/mL. The cells pellets were obtained by centrifugation for 5 min at 675×*g*. The medium was replaced with 100 μL of freshly prepared NR solution (50 μg/mL of stock solution) added to each well, including the blanks (media combined with sample) and the controls (non-treated cells). The solution mixture was incubated at 37 °C in an incubator with 5% CO_2 for 2 h. After incubation, the cells were pelleted and the 100 μL of NR and medium was discarded. The cells were rinsed with 150 μL PBS (pH 7.4) then 200 μL of 0.33% HCl in isopropanol solution was added to each well (including the controls and blanks) followed by thorough mixing. Finally, the absorption of the solutions was measured at 520 nm and the % cell cytotoxicity calculated.

2.5. Determination of cellular melanin content

Melanin is the product of melanocytes that protected skin damages by UV but the excessive melanin production or abnormal distribution can cause hyperpigmentation. So, the action of sesame compounds in reduction of cellular melanin content was evaluated. Melanin content was tested in the SK-MEL2 cell line, which was cultured at 2×10^5 cells/well in 24-well plates incubated at 37 °C in 5% CO_2 for 24 h. Sesame compounds and the positive control (kojic acid and β-arbutin) were incubated in the cells for 48 h. The final concentration used were as follows: (10–50) μg/mL sesamin, (0.3–30.0) μg/mL sesamol, (10–50) μg/mL sesamolin, (6–600) μg/mL kojic acid and (100–1 000) μg/mL β-arbutin. Afterward, cells were washed with PBS, lysed with 300 μL of 1 N NaOH and incubated at 70 °C for 1 h. The absorbance of cell lysis was measured at 405 nm.

2.6. Determination of cellular tyrosinase inhibition activity

The inhibiting potency of sesame compounds on the cellular tyrosinase was examined in the SK-MEL2 cell model. The cellular tyrosinase inhibitory assay-referred to by Sapkota *et al.* [24] with some modifications-was performed. The SK-MEL2 cells at 9×10^5 cells/well in 6-well plates were cultured and incubated at 37 °C, 5% CO_2 for 24 h. Cells were treated with sesamin, sesamol, sesamolin or the positive controls (kojic acid and β-arbutin) with 48 h exposure times at the same concentration as that used in the melanin content method. After treatment, cells were washed with ice-cold PBS and lysed with 1 mL of PBS 0.1 M, pH 6.8, containing 1% Triton X-100, at 4 °C for 30 min. Lysed cells were centrifuged at 10 000×*g* for 10 min. The supernatant containing the tyrosinase enzyme was analyzed for protein content using the BCA protein assay kit with bovine serum albumin-the control protein. The supernatant containing the same amount of protein (60 μg) was added to each well of a 96-well plate. Then L-DOPA in 0.1 PBS (pH 6.8) was added to each well for a final concentration of 4.5 mM and incubated at 37 °C. Absorbance of L-dopachrome was measured at 475 nm and %inhibition calculated.

2.7. Protein expression using Western blot analysis

Decrease in the melanin content is related to intracellular levels of melanogenic enzymes expression; hence, expression of melanogenic proteins was evaluated. SK-MEL2 was cultured at 9×10^5 cells/well in 6 well plates and incubated at 37 °C in 5% CO_2 for 24 h. Cells were treated with sesame compounds and the whitening agents-kojic acid and β-arbutin at 50 μg/mL for 48 h. Afterward, treated cells were washed with ice-cold PBS. Washed cells were lysed with lysis buffer and centrifuged at 10 000×*g* for 10 min. The supernatant was determined for protein content using a BCA protein assay kit. Next, the protein was

separated by 10% SDS-PAGE and transferred to a polyvinylidine difluoride membrane. The membrane was blocked with blocking solution at room temperature (26 °C) for 60 min, then incubated with primary antibody for tyrosinase, TRP1, TRP2 and β-actin overnight at 4 °C. After washing with TBS-T, the membrane was incubated with horseradish peroxidase, conjugated anti-goat IgG_1 at room temperature for 60 min. After washing with TBS-T, the bands were detected using an Amersham biosciences ECL kit. Finally, the chemiluminescent signal was detected by 5 min exposure using an Image Quant 400 machine. The bands were compared using a Scion Image program (version: 4.0.2).

2.8. Statistical analysis

The data from this study were expressed as means ± SD (n = 3). Statistical differences among the treated and untreated groups were analyzed using a one-way ANOVA with a 95% confidence interval.

3. Results

3.1. Effect of sesame compounds on sunscreen function

Different types of UV radiation have different mechanisms of cell toxicity. UVB radiation (290–320) nm possesses phototoxic effects, causing erythema, sunburns, immune suppression and initiation of photo-carcinogenesis. UVA radiation (320–400) nm induces oxidative stress, membrane permeability and lipid peroxidation. All of these deleteriously affect our skin. UVB is moreover 1 000 times more capable of causing sunburn than UVA, and it is more genotoxic. UVA, however, is 10 times more effective than UVB at causing lipid peroxidation [29].

The skin protects itself from UV exposure by increasing the amount of melanin pigment (resulting in skin darkening)-a putative physical sunscreen. Sunscreen agents can be applied topically on the skin as an extrinsic way to avoid increasing the melanin pigment. The search for alternative natural agents for UV protection gains more interest. Therefore, the objective of this study was to investigate the sunscreen function of sesamin, sesamol, and sesamolin. The UV profile of sesamin, sesamol, and sesamolin and standard anti-melanogenic agents (kojic acid and β-arbutin) were scanned for their absorbance (Figure 1). This assay was based on the assumption that the compound that absorbs UV in the respective UVA or UVB range will have a sunscreen effect. Our results showed that all three compounds absorbed ultraviolet in the UVB range. Sesamin and sesamolin primarily absorbed UVB, which both showed absorbance intensity (Abs) around 1.3 at 290 nm. Kojic acid (Abs of 0.2 at 300 nm) and β-arbutin (Abs of 0.2 at 300 nm) possessed about 4 folds lower absorbance (or lower sunscreen functions) than sesamol (Abs of 0.8 at 300 nm) (Figure 1).

3.2. Inhibition of mushroom tyrosinase in vitro

The anti-melanogenic activity of sesamol was evaluated by its inhibition of mushroom tyrosinase activity *in vitro*. The results showed that sesamol and kojic acid inhibited tyrosinase activity in a concentration-dependent manner. The respective IC_{50} value for sesamol and kojic acid was 0.33 μg/mL (1.6 μM) and 6.15 μg/mL (67.6 μM) (Figure 2B and 2D). Sesamin and sesamolin also showed slight tyrosinase inhibition activity. The other positive control (β-arbutin) did not show any tyrosinase

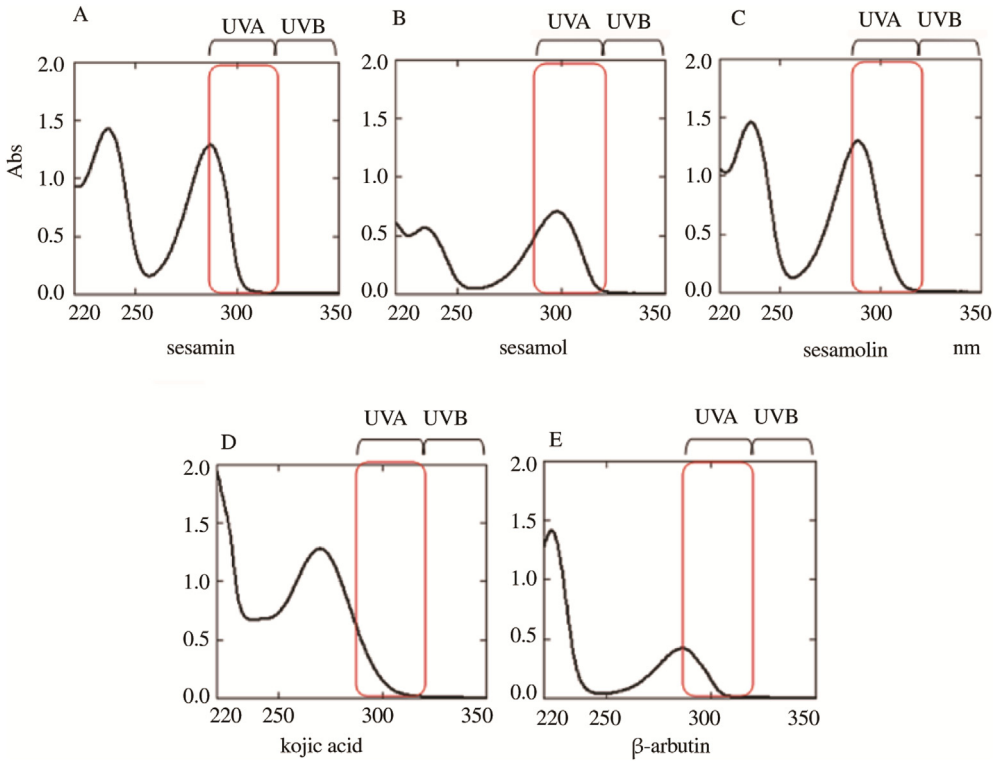

Figure 1. UV profiles of 3.33 mM of sesamin (A), sesamol (B), sesamolin (C), kojic acid (D), β-arbutin and (E) in methanol. Respective UVA and UVB absorbance between 320–400 nm and 290–320 nm.

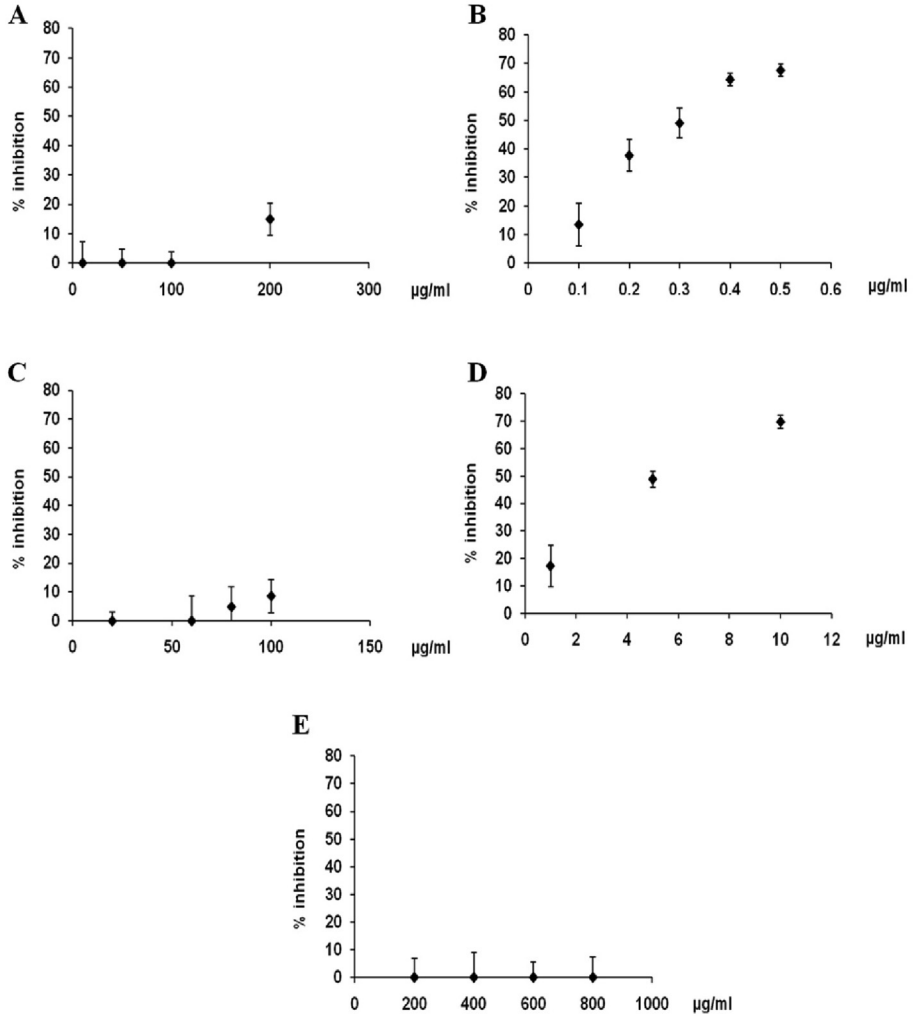

Figure 2. Effect of sesamolin, sesamin, sesamol, kojic acid and β-arbutin on mushroom tyrosinase inhibition.
(A), (10–200) µg/mL sesamin, (B), (0.1–0.5) µg/mL sesamol, (C), (20–100) µg/mL sesamolin, (D), (1–10) µg/mL kojic and (E), (200–800) µg/mL β-arbutin. Data were represented as mean from triplicate determinations.

inhibitory activity, despite using a concentration as high as 800 µg/mL (2 938.5 µM), indicating that sesamol had the greatest blocking effect on the tyrosinase enzyme, even more than the known whitening compounds, kojic acid and arbutin.

3.3. Cell cytotoxicity

To test the possibility of a clinical use for sesamol as a skin application, the cytotoxicity of sesamol was investigated *in vitro* in both normal (Vero) and SK-MEL2 cell lines. The Vero cell line was used to represent normal cells. Cytotoxicity was evaluated using a colorimetric NR assay [29]. Viable cells accumulate and bind neutral red within the lysosomes, but dead cells cannot because of the fragility of the lysosomal membrane and irreversible molecular alterations in the dead cells [28]. Figure 3 shows a line plot of % cytotoxicity versus concentrations of sesame compounds and positive skin-whitening agents. After 48 h of treatment, none of the compounds demonstrated any serious toxicity to the SK-MEL2 and Vero cell lines, excepted sesamol vis-à-vis the SK-MEL2 cell line. Although as much as 800 µg/mL sesame compounds were used, they barely affected cell viability. The cytotoxicity of sesamol to the Vero cells was only 22.8% after 48 h treatment. Similar non-cytotoxicity to the Vero cell line was observed for β-arbutin and kojic acid, which possessed a cytotoxicity of only 6.1% and 7.9%, respectively. However, the dimethyl sulfoxide was used to dissolve the test compounds when prepared the stock solution, therefore, dimethyl sulfoxide might be attributed to cytotoxicity.

The SK-MEL2 cell line was used to represent melanocytes. The cytotoxicity of the test compounds on the growth of SK-MEL2 was non-cytotoxic when using concentrations was up to 800 µg/mL (Figure 3). Cytotoxicity of between 42% and 97% was observed in the sesamol-treated SK-MEL2 cell line, at concentrations ranging between (600–800) µg/mL (Figure 3), while β-arbutin and kojic acid did not possess any cytotoxicity. Sesamol thus possesses slight cytotoxicity with an IC_{50} value of 608.93 µg/mL (4 406.5 µM) in the SK-MEL2 cell line (Figure 3). Since sesamin and sesamolin do not dissolve well in aqueous or media, the maximum concentrations of sesamin and sesamolin used in the study were based on their highest solubility (10–50) µg/mL, which had no cytotoxicity in either cell line.

3.4. Inhibition of cellular tyrosinase inhibition

The cellular tyrosinase inhibitory activity was evaluated in the SK-MEL2 cell model. The concentration used in the cell-based assay was higher than that used in the inhibitory study of mushroom tyrosinase *in vitro*, in order to ensure sufficient accumulation

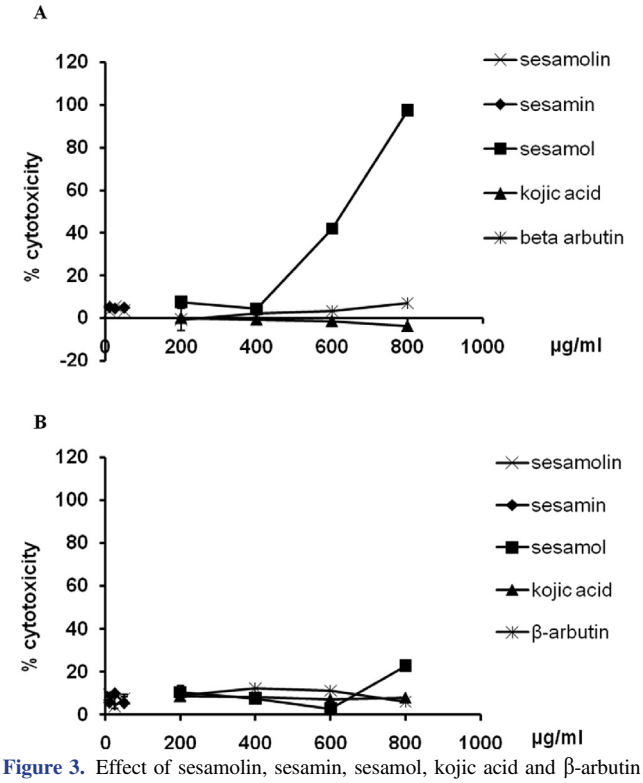

Figure 3. Effect of sesamolin, sesamin, sesamol, kojic acid and β-arbutin on cytotoxicity of the (A) SK-MEL2 and (B) Vero cell lines after 48 h treatment.
Data are presented as mean ± SD (n = 3).

of the test compound in the cells. The concentrations used were as follows: (10–50) μg/mL sesamin, (0.3–30.0) μg/mL sesamol, (10–50) μg/mL sesamolin, (6–600) μg/mL kojic acid and (100–1 000) μg/mL β-arbutin. However, sesamin and sesamolin had limit solubility under the condition studied. Thus, the maximum concentration for sesamin and sesamolin used was 50 μg/mL. The results of cellular tyrosinase activity are presented in Figure 4. Sesame compounds and kojic acid demonstrated similar tyrosinase inhibitory activity while β-arbutin did not, despite its being a positive control. Sesamolin (50 μg/mL) showed higher tyrosinase inhibition 50.00% ± 4.35% than sesamol (30 μg/mL, 23.55% ± 8.25%), sesamin (50 μg/mL, 23.14% ± 4.95%), kojic acid (600 μg/mL, 33.88% ± 1.43%) and β-arbutin (1 000 μg/mL, 8.26% ± 8.67%), respectively. Sesamolin exhibited the highest cellular tyrosinase inhibition. Although kojic acid was used at a higher concentration (600 μg/mL) than sesamol (30 μg/mL) and sesamin (50 μg/mL), kojic acid showed tyrosinase inhibitory activity not significantly different than sesamol or sesamin. By comparison, even though β-arbutin was as high as 1 000 μg/mL, the inhibition of cellular tyrosinase enzyme activity was < 10% (Figure 4).

3.5. Melanin content

Disruption of melanogenesis by natural depigmentation agents has been targeted for therapies or protection from hyperpigmentation disorders. Decreasing melanin content in melanocyte cell lines was an approach taken in the search for skin lightening. In the

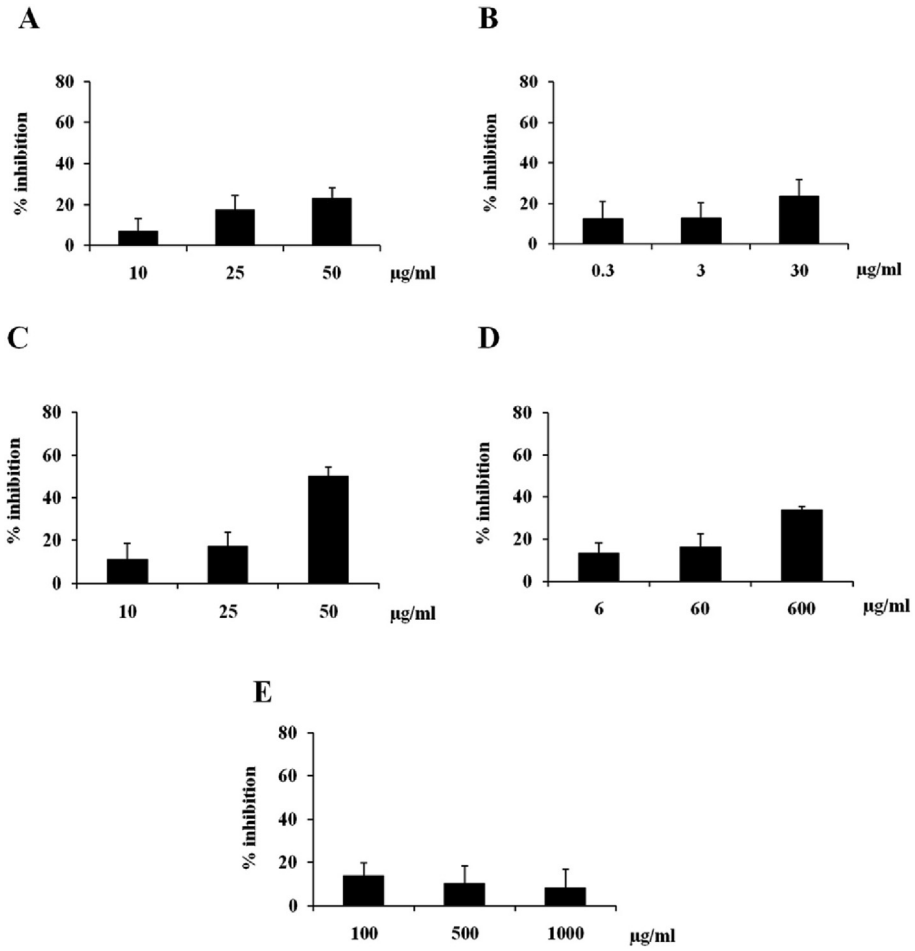

Figure 4. Effect of sesamin, sesamol, sesamolin, kojic acid and β-arbutin on cellular tyrosinase inhibition.
(A), 10–50 μg/mL sesamin, (B), 0.3–30.0 μg/mL sesamol, (C), 10–50 μg/mL sesamolin, (D), 6–600 μg/mL kojic and (E), 100–1 000 μg/mL β-arbutin. Data were represented as mean from triplicate determinations.

current study, a colorimetric assay of melanin content was used and the results showed inhibition of melanin production by sesamin, sesamol, sesamolin and the positive controls (kojic acid and β-arbutin). The high to low inhibition of melanin pigment was detected by sesamolin, sesamin, sesamol, kojic acid, and β-arbutin, respectively (Figure 5). Sesamolin at 25 μg/mL inhibited greater melanin content (41.67% ± 1.67%) than sesamin (25 μg/mL, 32.78% ± 2.89%). Sesamol at 30 μg/mL inhibited melanin content for only 7.77% ± 1.66%. Kojic acid had lesser % inhibition than sesamol (7.78% ± 0.96%) even using higher concentration (60 μg/mL). β-arbutin at 100 μg/mL inhibited the melanin content by only 12.78% ± 2.55%. The decrease in melanin pigment by sesame compounds was, moreover, more effective than β-arbutin.

3.6. Melanogenic protein expression assessed by Western blot analysis

It is well established that there are not only tyrosinase but also additional number of other proteins (TRP-1 and TRP-2) which are also associated with melanogenesis. Therefore, we tested the levels of these proteins by Western blot analysis after expose to sesame compounds. The expression of melanogenic protein (tyrosinase, TRP-1 and TRP-2) and the internal control (β-actin) were observed from the chemiluminescent signal and detected using an Image Quant 400 machine. We compared the band density between untreated and treated cells. The tyrosinase, TRP-1 and TRP-2 bands were observed (Figure 6). The band density of TRP-1 and TRP-2 proteins in the sesamolin-treated SK-MEL2 cells were 0.64 and 0.85, respectively. The results illustrated that sesamolin clearly reduced 36% TRP-1 and 15% TRP-2 protein levels, respectively compared to the control. Sesamin decreased only TRP-1 protein level showing band density of 0.84 (16.00% reduction compared to the untreated cells). Sesamol showed insipid depletion of both proteins. Tyrosinase protein was not decreased in the SK-MEL2 cells treated with the sesame compounds, kojic acid or β-arbutin. It is likely that the compounds did not affect tyrosinase protein expression.

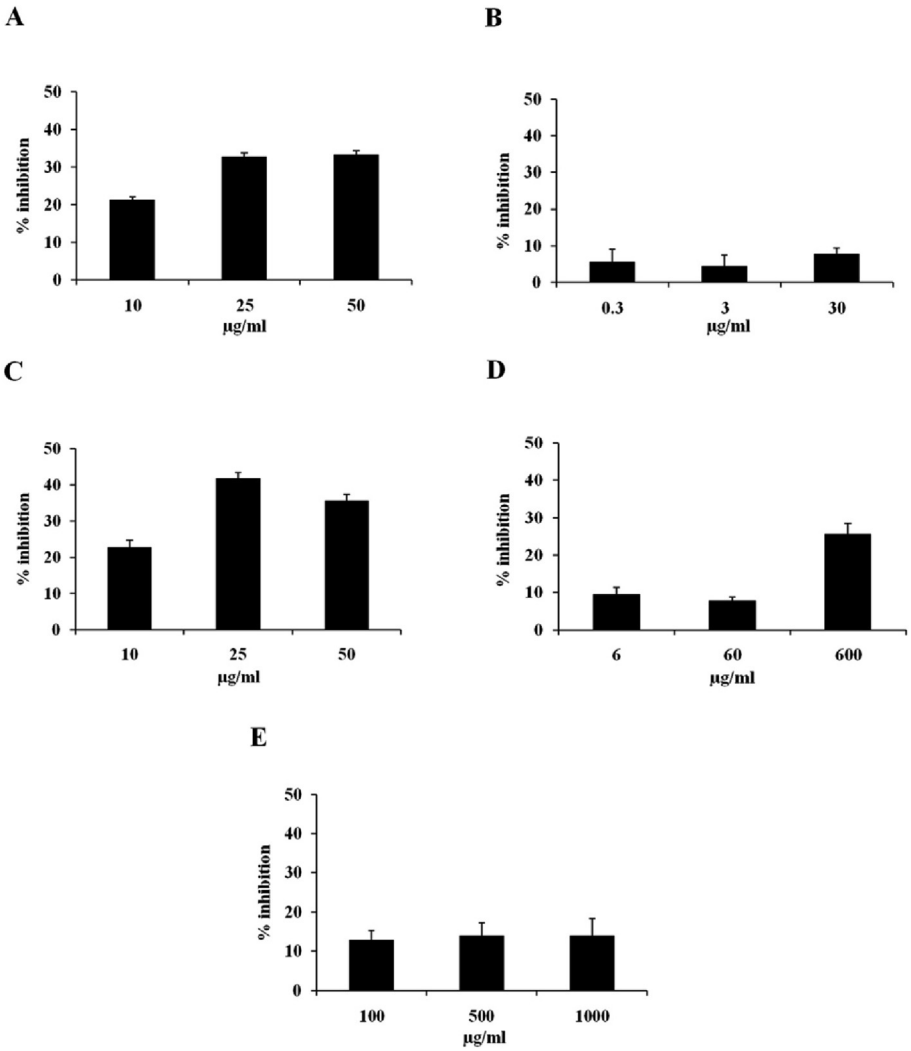

Figure 5. Effect of sesamin, sesamol, sesamolin, kojic acid and β-arbutin on inhibition of melanin content in SK-MEL2.
(A), 10–50 μg/mL sesamin, (B), 0.3–30.0 μg/mL sesamol, (C), 10–50 μg/mL sesamolin, (D), 6–600 μg/mL kojic acid and (E), 100–1 000 μg/mL β-arbutin. Data were represented as mean ± SD.

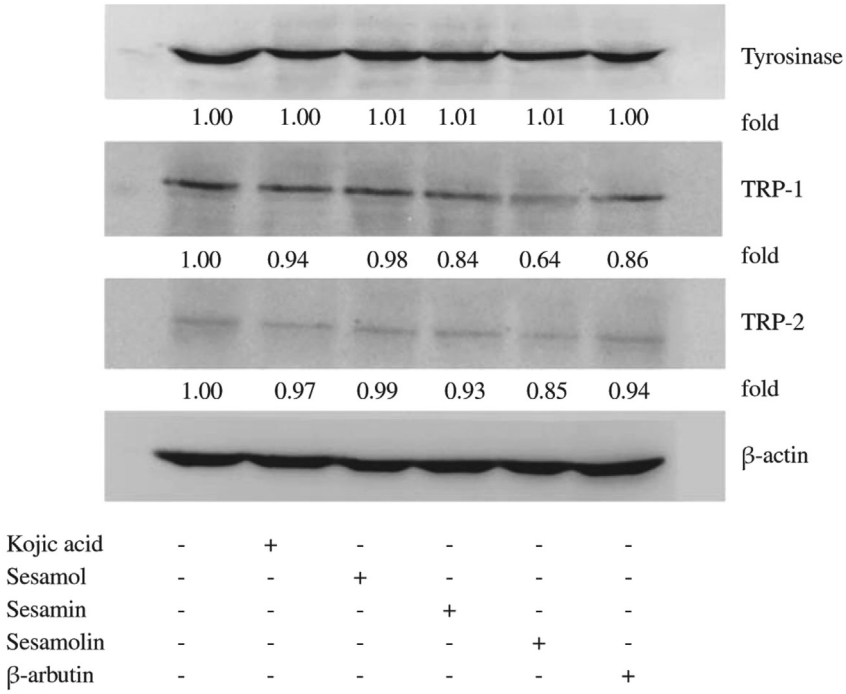

Figure 6. Effect of sesame compounds on the expression of melanogenic proteins in SK-MEL2 melanoma cells by Western blotting analysis. SK-MEL2 cells were treated with sesame compounds and antimelanogenic agent (kojic acid and β-arbutin) at 50 μg/mL for 48 h. β-actin was used as a protein-loading control. Band intensities were quantified based on the change of protein expression level.

4. Discussion

The anti-melanogenic effect of sesame compounds-sesamin, sesamol and sesamolin-from the seed of *Sesamum indicum* L. was investigated, through (a) the sunscreen function test, (b) the tyrosinase activity assay, (c) the melanin content assay and (d) melanogenic protein expression (tyrosinase, TRP-1 and TRP-2) using Western blot assay.

The first step for skin protection from UV radiation requires photo-protection (via a sunscreen function) to prevent melanin production. UV opacity is the property of sunscreens to absorb and/or block UV radiations, and thus to protect the skin from damage. In order to obtain photoprotection, topical strategies must protect against each range of UV radiation. This function can protect cells or biomolecules from UV-associated disease and reduction of melanin synthesis, leading to skin lightening [5]. In this study, UV absorbing properties of sesame compounds were evaluated by using UV spectral scanning function covering UV regions. In the current study, all three sesame compounds were found to fully absorb the UVB range, almost to 1.4 AU (around 290 nm), which possessed a higher absorbance (sunscreen function) than β-arbutin or kojic acid. Sesame compounds showed UV absorbing properties and would require further investigation to estimate the potential of sesame compounds as a photoprotection from skin damage or possibly for further developing UV protective products.

Sesame compounds were shown to possess tyrosinase-inhibitory effects. Sesamol and kojic acid inhibited mushroom tyrosinase activity *in vitro* in a concentration-dependent manner. Sesamol was the strongest mushroom tyrosinase inhibitor. Sesame compounds and the positive control were further tested for their anti-tyrosinase activity in the melanoma SK-MEL2 cell line to confirm the cellular inhibitory activity of tyrosinase enzyme. The concentration used in the cell-based assay was higher than that used in the *in vitro* inhibitory study of mushroom tyrosinase, in order to ensure sufficient uptake of the test compounds into the cells. At the maximum concentration used, sesamolin (50 μg/mL) exhibited the highest cellular tyrosinase inhibition (50.00% ± 4.35%). Although kojic acid was used at a higher concentration (600 μg/mL) than sesamol (30 μg/mL) and sesamin (50 μg/mL), kojic acid showed 33.88% ± 1.43% tyrosinase inhibitory activity, which was not significantly different from sesamol 23.55% ± 8.25% and sesamin 23.14% ± 4.95% ($P < 0.05$). Even though β-arbutin was used concentrations as high as 1 000 μg/mL, its inhibitory effect on cellular tyrosinase enzyme was < 10%.

Sesamolin, sesamin and sesamol were more effective anti-melanogenic agents than the known anti-melanogenic compounds, kojic acid and β-arbutin. Sesamin and sesamolin had negligible inhibitory mushroom tyrosinase effect but they showed high inhibition on cellular tyrosinase activity, perhaps due to the high lipophilicity of sesamolin and sesamin over against sesamol, leading to more intracellular uptake. The lipophilicity of sesamolin, sesamin and sesamol, represented as a log octanol/water, is 3.49, 3.45, and 1.29, respectively [30–32].

The cellular transformation of sesamolin and sesamin to phenol- or catechol-containing compounds was reported. The metabolites of sesamolin and sesamin might interrupt the active binding site of tyrosinase [33,34]. β-arbutin was previously reported to have no inhibition effect on mushroom tyrosinase but an inhibitory effect against melanoma tyrosinase [35]. The cellular tyrosinase inhibition of β-arbutin is suggested to be due to the intracellular conversion of arbutin to hydroquinone via the hydrolysis reaction by bacteria on the skin of humans [36]. The glycosidic linkage of the arbutin structure might be cleaved in the cellular assay resulting in the tyrosinase inhibitory activity [37].

The enzymatic cascade that plays an important role in melanin synthesis is controlled by tyrosinase, TRP-1, and TRP-2 [38]. The inhibition of tyrosinase, TRP-1 and TRP-2 protein expression leads to hypopigmentation, which is a desired

property in depigmenting agents and cosmetics. To elucidate the effect of sesame compounds on melanogenic protein expression in a SK-MEL2 cell model, the same concentration (50 μg/mL) of compounds was used. Western blot assay showed that sesamolin suppressed the expression of both TRP-1 and TRP-2 protein levels, whereas only sesamin and β-arbutin suppressed the TRP-1 level. All three sesame compounds were, however, not able to suppress tyrosinase protein expression at 50 μg/mL. Likewise, known skin lightening agents (*i.e.*, from *Arthrophytum scoparium*, *Glechoma hederacea* and nicotinic acid hydroxamate) are reported to suppress either one or both anti-melanogenic proteins; *viz.*, tyrosinase (*G. hederacea, A. scoparium* and nicotinic acid hydroxamate) and TRP-1 (*A. scoparium*, nicotinic acid hydroxamate). Nicotinic acid hydroxamate and *G. hederacea* were not found to suppress TRP-2 in B16F10 melanoma cells nor TRP-1 and TRP-2 in B16 melanoma cells [39–41].

Tyrosinase activity depends on the binding and function of two copper atoms at the active site. Upon binging with the inhibitor, decreasing of tyrosinase activity is occurred leading to depigmentation. The action of inhibitor on tyrosinase can be via competitive inhibition and non-competitive inhibition. Moreover, copper chelation at active site is also reducing tyrosinase activity [42]. Decrease in the intracellular levels of tyrosinase and other melanogenic enzymes expression are related to tyrosinase transcription, tyrosinase degradation and may be tyrosinase glycosylation and obstruction of other melanogenic enzymes [42]. Therefore, inhibition of tyrosinase without detection of tyrosinase expression in cells treated with sesame compounds kojic aid and β-arbutin may involve with other mechanism and that is also challenged for further evaluation.

The inhibition of cellular melanin content was also detected, confirming that these sesame compounds possess skin-lightening activity. The order of melanin inhibition effect from high to low is sesamolin > sesamin > sesamol > kojic acid > β-arbutin. Kojic acid at the maximum concentration (600 μg/mL) showed a greater inhibition of melanin content 25.55% ± 2.88% than 30 μg/mL sesamol 7.77% ± 1.66%. For kojic acid to exert a similar inhibition on melanin content as sesamol (7.8%), the concentration had to be 2 times higher (60 μg/mL) than sesamol (30 μg/mL), which evidences that sesamol possesses a stronger anti-melanogenic activity than kojic acid.

Melanin synthesis and deposition is regulated via 3 stages: before, during and after melanin synthesis. Melanin synthesis inhibitions can, therefore, proxy as a prevention process. Melanin biogenesis requires a melanin-inducer (*e.g.*, UV radiation) and a melanogenic enzyme (*i.e.*, tyrosinase, TRP-1 and TRP-2). The loss of these 3 factors will lead to a reduction in the production of melanin pigments. Sesame compounds prevented melanin synthesis through 2 stages: (a) by blocking melanin-induction and (b) by interrupting melanogenic enzyme production. Our data confirm that sesamolin, sesamin and sesamol have a photo-protective effect (sunscreen function) in the range of UVB-the melanin synthesis stage. Sesamolin showed the highest UVB absorption while sesamolin and sesamin suppressed expression of the melanogenic proteins TRP-1/TRP-2 and TRP-1, respectively. During melanin synthesis, the biochemical data illustrate that sesamolin, sesamin and sesamol possessed anti-melanogenesis via inhibition of the tyrosinase activity, leading to a decrease in melanin content. Melanin synthesis was thus inhibited or delayed, leading to a skin-lightening effect. The last step-related to inhibition of melanosome transfer and acceleration of skin turnover-occurred after melanin synthesis and should be further evaluated. It is interesting that each sesame lignan plays different roles in anti-melanogenesis via multiple mechanisms of action and to different extents. Sesamolin, sesamin and sesamol, therefore, represent potential cosmeceuticals.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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