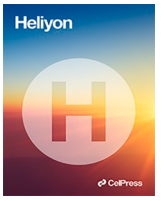
Heliyon 10 (2024) e33423

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Anti-tyrosinase and anti-melanogenic effects of piperine isolated from *Piper nigrum* on B16F10 mouse melanoma cells

Phanthiwa Khongkarat [a,1], Ponglada Sadangrit [a,1], Songchan Puthong [b], Thitipan Meemongkolkiat [a], Preecha Phuwapraisirisan [c], Chanpen Chanchao [a,*]

[a] *Department of Biology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok, 10330, Thailand*
[b] *Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, 254 Phayathai Road, Bangkok, 10330, Thailand*
[c] *Center of Excellence in Natural Products, Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok, 10330, Thailand*

ARTICLE INFO

Keywords:
B16F10 melanoma cells
Melanin content
Melanogenesis-related genes
Piperine
tyrosinase inhibitory activity

ABSTRACT

The essential function of melanin is to protect our skin against harmful environmental factors. However, excessive melanin production can cause undesirable hyperpigmentation issues, such as freckles and melasma. Although several compounds are used to control melanin production by inhibiting tyrosinase (TYR), their efficacy is limited by skin-related adverse effects and cytotoxicity concerns. Consequently, searching for new natural compounds with an effective TYR inhibitor (TYR-I) activity but less harmful effects continues. Plant-based natural extracts are an alternative that are in great demand due to their safety and diverse biological properties. This study assessed ten isolated plant compounds for their TYR-I activities using an *in vitro* mushroom TYR inhibition assay. Among these compounds, piperine (400 μM) demonstrated the highest TYR-I activity, with a potency of 36.27 ± 1.96 %. Hence, this study examined the effect of piperine on melanogenesis in melanocyte stimulating hormone-treated B16F10 melanoma cells and using kojic acid as a positive reference. Cell viability was evaluated through the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Measurements of cellular TYR activity and melanin content were performed and related to changes in the transcriptional expression levels of melanogenesis-related genes, assessed via quantitative real-time reverse transcriptase (RT-q)PCR analysis. The results revealed that piperine at a concentration of 44 μM significantly reduced cellular TYR activity by 21.51 ± 2.00 % without causing cytotoxicity. Additionally, at the same concentration, piperine significantly decreased the intracellular melanin content by 37.52 ± 2.53 % through downregulating transcription levels of *TYR* and TYR-related protein 1 (*TRP-1*) but not *TRP-2*. Kojic acid, at a concentration of 1407 μM, induced a significant

Abbreviations: TYR, tyrosinase; TYR-I, tyrosinase inhibitor; TRP-1, tyrosinase-related protein 1; TRP-2, tyrosinase-related protein 2; RT-qPCR, quantitative reverse transcription polymerase chain reactions; MSH, α-melanocyte stimulating hormone; IC_{50}, half maximal inhibitory concentration; UVR, ultraviolet radiation; HQ, hydroquinone; DMSO, dimethyl sulfoxide; L-DOPA, L-3,4-dihydroxyphenylalanine; CM, culture medium; DMEM, Dulbecco's modified Eagle Medium; EDTA, ethylene diamine tetra-acetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviation; ANOVA, one-way analysis of variance; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; MITF, microphthalmia-associated transcription factor; –OH, hydroxy.

* Corresponding author.
E-mail addresses: parephanthiwa@gmail.com (P. Khongkarat), negi_ponglada@hotmail.com (P. Sadangrit), songchan.p@chula.ac.th (S. Puthong), lek_tues_ti@hotmail.com (T. Meemongkolkiat), preecha.p@chula.ac.th (P. Phuwapraisirisan), chanpen.c@chula.ac.th (C. Chanchao).
[1] Shared first authors.

https://doi.org/10.1016/j.heliyon.2024.e33423
Received 12 March 2024; Received in revised form 4 June 2024; Accepted 21 June 2024
Available online 22 June 2024
2405-8440/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

decrease in the melanin content and cellular TYR activity by suppressing all three melanogenesis-related genes. These findings suggest that piperine has potential as a potent depigmenting agent.

1. Introduction

Melanin, a biological pigment, is commonly present in various organisms. It is synthesized and stored within melanosomes, organelles found among melanocytes in the basal layer of the skin epidermis, and is later transferred to adjacent keratinocytes through the physiological process called melanogenesis [1]. Melanin plays an important role in the protection against the harmful effects of ultraviolet radiation (UVR), oxidative stress, DNA damage, and various environmental stressors as well as in determining the color of skin, hair, and eyes [2]. The regulation of melanogenesis is predominantly determined by genetics but can be influenced by numerous external and internal variables, including hormonal fluctuations, growth factors, cytokines, medications, inflammation, age, and exposure to UVR [3,4]. All these stimuli trigger the microphthalmia-associated transcription factor gene, which in turn regulates the expression of genes involved in melanin synthesis, including tyrosinase (*TYR*), TYR-related protein 1 (*TRP-1*), and *TRP-2* [5]. The process begins with the hydroxylation of L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA), followed by L-DOPA oxidation into DOPA quinone. Notably, within this oxidation step, TYR functions as the rate-limiting enzyme in the overall melanin biosynthesis pathway, including the formation of eumelanin and pheomelanin, which are responsible for the brown-black and red-yellow colors, respectively, while TRP-1 and TRP-2 are closely linked to only eumelanin biosynthesis [2].

While melanin plays a vital role in protecting the skin against various environmental factors, excessive melanin production or hyperpigmentation can lead to dermatological issues, including age spots, freckles, melasma, senile lentigo, pigmented acne scars, as well as post-inflammatory hyperpigmentation caused by atopic dermatitis and melanoma, which are global public health concerns in both pediatric and adult populations [6–8]. Consequently, researchers have focused their efforts on targeting TYR inhibitors (TYR-Is) as the primary approach to regulate melanin production. Numerous compounds, derived from both natural and synthetic sources, have been reported as TYR-Is, such as hydroquinone (HQ), ascorbic acid, arbutin, ellagic acid, tranexamic acid, and kojic acid [9]. However, the use of these inhibitors has raised concerns due to their associated side effects. For instance, kojic acid has been linked to contact dermatitis, carcinogenicity, and increased UV sensitivity problems [10]. Whereas, HQ exhibits toxicity towards mammalian cells, resulting in issues like contact dermatitis and irritation [11,12]. As an alternative, the use of TYR-Is from natural sources has garnered increasing attention, driven by consumer preference for natural ingredients, particularly plant extracts in skincare, to avoid potentially harmful synthetic compounds [13,14]. Hence, there is an imperative need to explore novel TYR-Is derived from natural sources that offer enhanced TYR-I efficacy while ensuring safety and minimizing harmful effects.

Plants represent a prominent source for the pursuit of TYR-Is, owing to their diverse array of secondary metabolites, especially, triterpene glycosides, flavonoids, phenolic compounds, alkaloids, chalcones, and lignans [14–17]. In this study, specific plant compounds in those arrays were selected for screening for TYR-I activity due to the intriguing presence of hydroxy (–OH) groups, aliphatic rings, or aromatic rings within their chemical structures, features that have been associated with their potential as TYR-Is. The –OH group can be chelated with dinuclear copper and also interacted with amino acid moieties in the TYR active site via hydrogen bonding [18]. In addition, the aromatic ring provides a π-π interaction with histidine residues in the TYR pocket. Although triterpenoid glycosides lack an aromatic moiety, the aliphatic ring of this array serves a hydrophobic interaction with the TYR pocket [19]. Furthermore, these selected compounds have previously demonstrated a multitude of biological properties. For example, alpinetin (**1**) demonstrates anticancer properties [20], cardamomin (**2**) has been reported to have anti-inflammatory, antineoplastic, and hypoglycemic properties [21], lansioside derivatives (**3**–**5**) display antioxidant and antimicrobial activities [22,23], malabaricone A (**6**) shows potential in antidiabetic applications [24], piperine (**7**) is known for its antiobesity, hepatoprotective, and anticancer properties [25], pinocembrin (**8**) exhibits anti-inflammatory effects [26], samin (**9**) was reported to form the core structure in the synthesis of antidiabetic agents [27], and sesamin (**10**) is associated with antinociceptive effects [28]. However, there is limited prior research on the TYR-I activities of compounds **1**–**10**.

This study focused on investigating the TYR inhibitor (TYR-I) activity of compounds **1**–**10**, which were isolated from five distinct plant sources. The primary screening for TYR-I activity was conducted using an *in vitro* mushroom TYR assay. After that, the most promising compounds underwent further evaluation for their anti-melanogenic activities at the cellular level, employing α-melanocyte stimulating hormone (MSH)-induced B16F10 melanoma cells as a model system. In addition, the *in vitro* toxicity of active compounds against B16F10 cells was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and changes in cell morphology were observed via light microscopy. In addition, the TYR-I activity and melanin production were also evaluated and correlated to alterations in the transcript expression levels of melanogenesis-associated genes using one-step quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR). If the results proved favorable, the TYR-I compound(s) may hold potential for applications in the cosmetic and pharmaceutical industries.

2. Materials and methods

2.1. Plant compounds preparation

Ten pure compounds, originating from five different plant sources, were provided by Dr. Preecha Phuwapraisirisan and Dr. Rico Ramadhan. These compounds are alpinetin (**1**), cardamomin (**2**), and pinocembrin (**8**) from *Boesenbergia rotunda*; lansioside B (**3**),

lansioside C (**4**), and methyl lansioside C (**5**) from *Lansium parasiticum*; malabaricone A (**6**) from *Horsfieldia motley*; piperine (**7**) from *Piper nigrum*; and samin (**9**) and sesamin (**10**) from *Sesamum indicum*. The chemical structures of these compounds are depicted in Fig. 1.

Subsequently, the tested compounds were dissolved in dimethyl sulfoxide (DMSO) and maintained at −20 °C as the stock solution.

2.2. In vitro mushroom TYR-I activity assay

The TYR-I test was conducted as previously described [29] using L-DOPA as the substrate to assess the efficacy of all plant compounds in inhibiting the diphenolase activity of mushroom TYR. The reaction mixture consisted of 120 μL of 1.5 mM L-DOPA in 80 mM phosphate buffer pH 6.8 (PB), 20 μL of PB, and 20 μL of the tested compounds at a concentration of 400 μM in DMSO. These mixtures were pre-incubated at 25 °C for 10 min. Subsequently, 40 μL of 165 units/mL mushroom TYR in 50 mM PB at pH 6.5 was added and incubated at 25 °C for 5 min. The absorbance was then measured at 475 nm (A_{475}) using a microplate reader (Thermo Fisher Scientific, USA). Kojic acid served as the positive reference inhibitor. Each sample was analyzed in triplicate, and the data is presented as the mean ± one standard deviation (SD). The TYR-I activity (%) was calculated using Eq. (1);

$$\% \text{ TYR-I} = \{[(A\text{-}B) - (C\text{-}D)]/ (A\text{-}B)\} \times 100, \quad (1)$$

where A is the A_{475} after incubation without the test substance, B is the A_{475} after incubation without the test substance and TYR, C is the A_{475} after incubation with the test substance and TYR, and D is the A_{475} after incubation with the test substance but without TYR.

2.3. Cell culture

B16F10 mouse melanoma cells (ATCC No. CRL-6475) served as the model and were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. These cells were cultured in culture medium [CM; Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) fetal calf serum (FCS) and 1 % (w/v) penicillin/streptomycin] in a 5 % (v/v) CO_2-humidified environment at 37 °C. Sub-culturing occurred every two days using a 0.05 % trypsin-EDTA solution when they reached 80 % confluence.

2.4. Cell viability assay

Cell viability was assessed using the MTT colorimetric assay as previously described [30], with slight modifications. B16F10 cells were initially seeded at a density of 1×10^4 cells per well in 200 μL of CM within 96-well plates and allowed to adhere for 24 h. Subsequently, the cells were exposed to 2 μL/well of one of the (i) respective test compound at different concentrations dissolved in

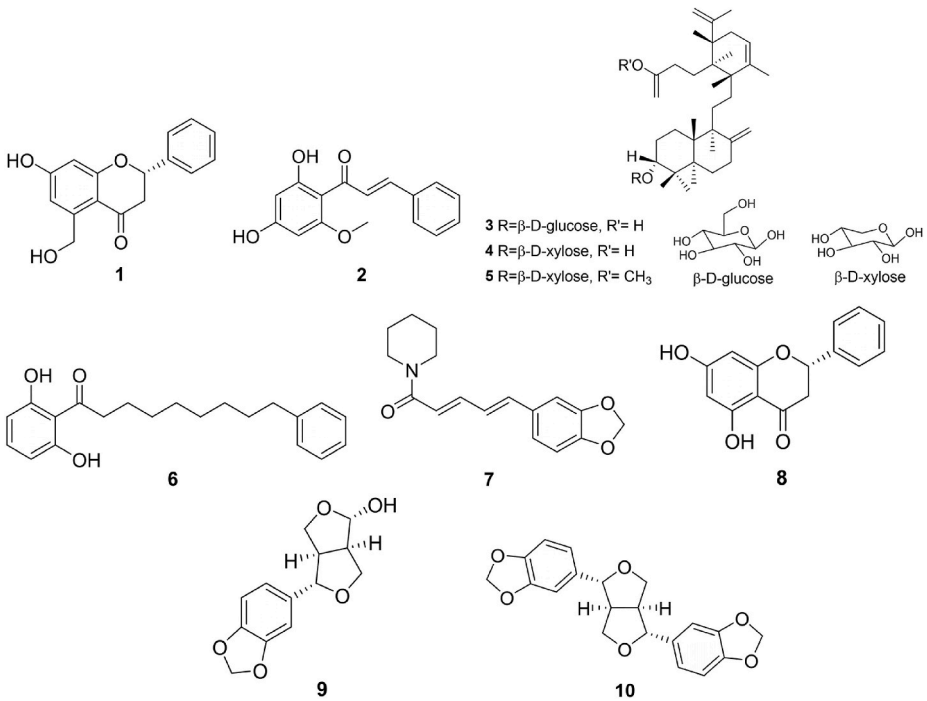


Fig. 1. The chemical structures of compounds **1**–**10**.

DMSO, (ii) kojic acid (positive), or (iii) DMSO as a negative control, resulting in a 1 % (v/v) final concentration of DMSO [31] in the presence of 10 nM α-MSH for 72 h. After the treatment period, the morphology of B16F10 cells in the treatment groups was observed under a light microscope at 200X magnification. To evaluate the relative cell viability (%), 10 μL of MTT solution (5 mg/mL in normal saline) was added to each well and incubated for 4 h to enable formazan production. Following incubation, the supernatant was carefully removed, and 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance at 540 nm (A_{540}) was recorded using a microplate reader. The results were expressed as the percentage relative cell viability (relative to the contro)l using Eq. (2).

$$\text{Relative cell viability (\%)} = (A_{\text{sample}}/A_{\text{control}}) \times 100, \quad (2)$$

where A_{sample} and A_{control} are the A_{540} values with and without the addition of test sample, respectively.

2.5. Cellular TYR activity assay

Cellular TYR activity was measured as previously described [32], with slight modifications. B16F10 cells were plated at a density of 1×10^6 cells per well in 6-well plates, each well containing 2 mL of CM, and were incubated for 24 h. Subsequently, the CM was replaced with fresh CM containing one of (i) various concentrations of the respective test compound, (ii) kojic acid, or (iii) 1 % (v/v) DMSO as a negative control, in the presence of 10 nM α-MSH for 48 h. Following this incubation period, the medium was aspirated, and the cells were washed twice with ice-cold phosphate buffered saline (PBS). Cell lysis was achieved using 400 μL of 0.01 M PB (pH 7.4) containing 1 % (v/v) Triton X-100 (lysis buffer). The mixture was freeze-thawed by incubating at −80 °C for 15 min and then thawed at room temperature for 10 min. After centrifugation at 12,000 g for 15 min at 4 °C, the supernatant was utilized to determine the protein concentration using the Bradford assay with bovine serum albumen as the standard. The protein concentrations were adjusted with lysis buffer. Subsequently, 100 μL of a freshly prepared substrate solution (5 mM L-DOPA in 50 mM pH 7.1 sodium phosphate buffer) was combined with 100 μL of lysate supernatant, each containing 100 μg of protein, in a 96-well plate. The reaction mixture was then incubated at 37 °C for 1 h, and the A_{475} was read using a microplate reader. The percentage of TYR-I activity was then calculated using Eq. (1).

2.6. Cellular melanin content

Melanin content was quantified as previously described [33], with slight modifications. B16F10 cells were seeded at a density of 5×10^4 cells per well in 6-well plates, each well containing 2 mL of CM, and then incubated for 24 h. The medium was then replaced with fresh CM containing one of (i) various concentrations of the respective test compound, (ii) kojic acid, or (iii) 1 % (v/v) DMSO as a negative control, in the presence of 10 nM α-MSH for 72 h. The extracellular melanin content (melanin secreted into the CM) was determined at a wavelength of 405 nm (A_{405}) by transferring 200 μL of the CM to a 96-well plate. Subsequently, the intracellular melanin content was determined after removing the CM, washing the cells three times with PBS, then adding 500 μL of 1 N NaOH containing 10 % (v/v) DMSO to solubilize the cell pellet and incubating at 80 °C for 1 h. The A_{405} was measured using a microplate reader. The relative melanin content was calculated from a synthetic melanin standard curve (ranging from 0 to 200 μg/mL). Melanin production was expressed as a percentage relative to the untreated controls.

2.7. RNA preparation and RT-qPCR

Transcript expression levels of the *TYR*, *TRP-1*, and *TRP-2* genes were determined using one-step RT-qPCR. B16F10 cells were seeded onto 6-well plates at a density of 1×10^6 cells per well in CM and allowed to attach overnight. Subsequently, the cells were treated with fresh CM containing various concentrations of the respective compound, kojic acid, and 1 % (v/v) DMSO as a negative control, in the presence of 10 nM α-MSH for 48 h. The cells were then washed with ice-cold PBS and scraped off using a cell scraper. After centrifugation at 1500×*g* for 5 min, the supernatant was discarded, and total RNA was extracted from the cell pellet using the RNeasy mini kit (Catalog No. 74104; Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The isolated RNA samples were quantified using a nanophotometer (NanoPhotometer NP80, Implen GmbH, Munich, Germany) and stored at −20 °C until the RT-qPCR analysis.

The RT-qPCR was conducted using the One-Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time; Catalog No. R086A; Takara, Tokyo, Japan). Each 10-μL reaction mixture contained 5 μL of 2x One-Step SYBR® RT-PCR Buffer IV, 0.25 μL of PrimeScript™ Enzyme Mix II, 0.5 μL of forward primer (10 μmol/L), 0.5 μL of reverse primer (10 μmol/L), and 20 ng of total RNA. The primer sequences used in this study were sourced from previous reports [34], with the transcript level of the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene serving as the internal standard. All primer sequences are listed in Table 1. PCR amplification was

Table 1
Targeted genes and primers used for their amplification by RT-qPCR.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
GAPDH	GGGCATCCTGGGCTACTCTG	GAGGTCCACCACCCTGTTGC
TYR	GGGCCCAAATTGTACAGAGA	ATGGGTGTTGACCATTGTT
TRP-1	GTTCAATGGCCAGGTCAGGA	CAGACAAGAAGCAACCCCGA
TRP-2	GCTTGGAGCAGCAAGACAAG	ATTACACAGTGTGACCCGGC

performed using a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was carried out at 50 °C for 2 min, followed by 95 °C for 10 min. The PCR amplification step consisted of 40 cycles at 95 °C for 15 s and 50 °C for 1 min [34]. All reactions were conducted in triplicate, and the data were analyzed using the $2^{-\Delta\Delta CT}$ method. The expression levels of the target genes were normalized to the expression level of the *GAPDH* gene and relative to the control group [35].

2.8. Statistical analysis

Data are presented as the mean ± SD, derived from three independent repeats in each experiment. Significant differences between groups were assessed using one-way analysis of variance (ANOVA). For pairwise multiple comparisons, Tukey's tests were applied. P-values <0.05 were considered statistically significant. All statistical analyses were performed using the IBM SPSS statistics version 29.0.1.0 for windows software.

3. Results

3.1. Primary screening of plant compounds

Ten compounds (**1**–**10**; Fig. 1 and Table 2) plus the positive control (Kojic acid; **11**) were tested for their TYR-I activity using an *in vitro* mushroom TYR inhibitory assay. The obtained A_{475} was converted to the % TYR-I activity, and the results are summarized in Table 2. At a concentration of 400 μM, piperine (**7**) exhibited a significant TYR-I activity of 36.27 ± 1.96 %, although it was over 2.4-fold less effective than kojic acid. Nevertheless, its TYR-I activity was still considerably better than that of the nine other plant compounds. For sesamin (**10**), the A_{475} could not be measured at a concentration of 400 μM due to precipitation in the reaction, but it displayed a TYR-I activity of less than 10 % at a concentration of 200 μM. Therefore, only piperine was selected for further study of its potential anti-melanogenic activity in the B16F10 cell model.

3.2. Effect of the piperine on cell viability

The cytotoxicity of B16F10 cells following treatment with piperine was assessed through cell morphology and relative cell viability using the MTT assay. After incubating B16F10 cells with increasing concentrations of piperine (ranging from 3 to 175 μM) for 72 h, the results showed that piperine at concentrations between 3 and 44 μM was not cytotoxic to the cells, with a relative cell viability exceeding 80 %. However, at concentrations of 88 and 175 μM, the relative cell viability was reduced to 46.65 ± 12.56 and 6.23 ± 0.64 %, respectively, (Fig. 2). These results were consistent with the observed impact of piperine on the B16F10 cell morphology (see below).

In the control group, B16F10 cells displayed a mix of spindle-shaped and epidermal-like cells, were in close contact with neighboring cells, and adhered to the surface of the well plate (Fig. 3A). The morphology and viability of cells exposed to 3–44 μM piperine were indistinguishable from those of the control cells (Fig. 3B–F), whereas at 88 μM the cells exhibited a loss of cell-cell contact and a reduced density (number) of living cells (Fig. 3G). At a concentration of 175 μM, B16F10 cells showed signs of shrinkage, irregular cell outlines, more dendrites, and a dramatic decrease in cell density (number) compared to the control (Fig. 3H). Therefore, a concentration range of 0–44 μM piperine was selected for further experiments.

In contrast, kojic acid, as reported in our previous study, exhibited significant reductions in cell viability and morphological changes at concentrations of up to 1407 μM after a 72-h treatment [29]. Consequently, kojic acid at a concentration of 1407 μM was chosen as the positive control for subsequent experiments. The cytotoxic IC_{50} values for piperine and kojic acid against B16F10 cells were 90.16 ± 4.497 and 10,764.90 ± 0.555 μM, respectively, indicating that piperine was significantly more cytotoxic (lower IC_{50} value) than kojic acid.

Table 2
Percentage of TYR inhibition of plant compounds **1**–**10** and kojic acid (**11**).

Compounds	Percentage TYR inhibition at 400 μM
Alpinetin (**1**)	6.90 ± 5.76^{c}
Cardamomin (**2**)	5.58 ± 7.15^{c}
Lansioside B (**3**)	6.27 ± 0.85^{c}
Lansioside C (**4**)	NI
Methyl lansioside C (**5**)	NI
Malabaricone A (**6**)	NI
Piperine (**7**)	36.27 ± 1.96^{b}
Pinocembrin (**8**)	14.56 ± 1.82^{c}
Samin (**9**)	5.39 ± 4.77^{c}
Sesamin (**10**)	ND
Kojic acid (**11**)	89.7 ± 1.11^{a}

Remark: Data are shown as the mean ± SD derived from three replicates. Means within a column with a different superscript letter are significantly different [$p < 0.05$; one-way ANOVA and Post Hoc (Tukey) test]. ND represents not determined; NI represents no inhibition.

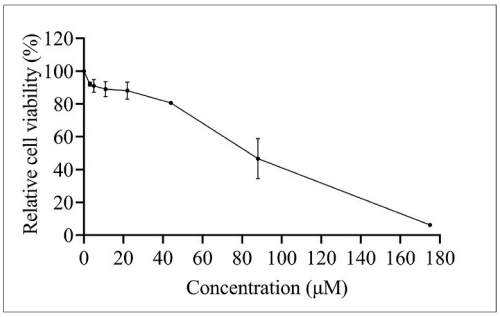


Fig. 2. Relative cell viability of B16F10 melanoma cells after treatment with piperine at various concentrations for 72 h, as determined by the MTT assay. Data are shown as the mean ± SD from three independent experiments.

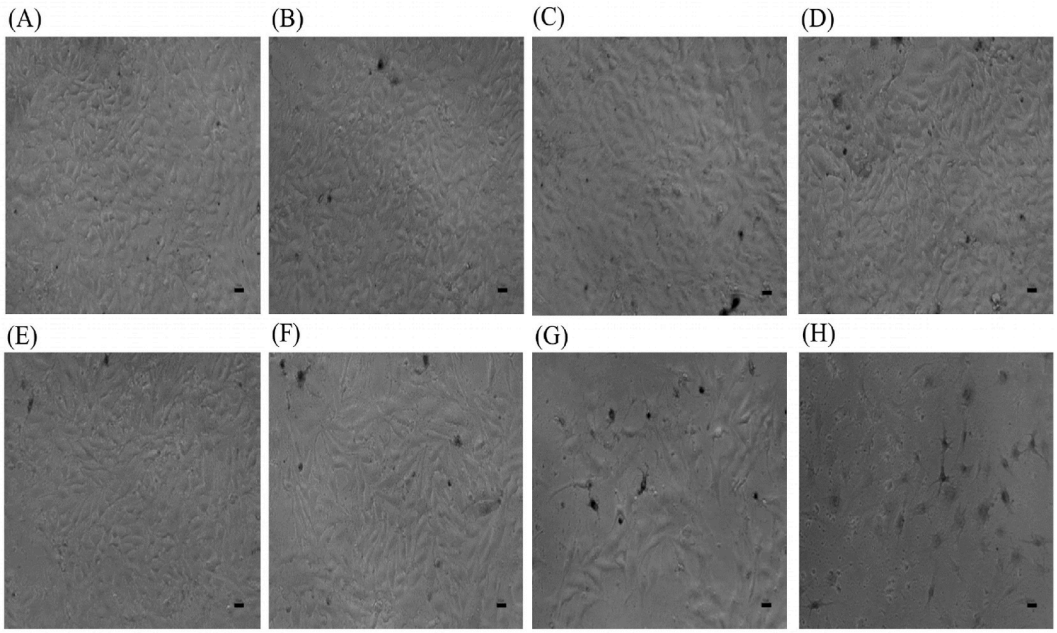


Fig. 3. Light microscopy (200X) images showing the morphology of B16F10 cells after exposure for 72 h to piperine at (A) 0 μM (control), (B) 3 μM, (C) 5 μM, (D) 11 μM, (E) 22 μM, (F) 44 μM, (G) 88 μM, and (H) 175 μM. Each picture is representative of three independent experiments. Scale bar = 10 μm.

3.3. Cellular TYR-I activity of piperine

The inhibitory effect of piperine on the TYR activity of α-MSH-stimulated B16F10 cells was examined. The B16F10 cells were incubated with non-cytotoxic concentrations of piperine (11, 22, and 44 μM) or kojic acid (1407 μM) for 48 h. As shown in Fig. 4, when comparing the cellular TYR activity to the control (set at 100 %), piperine demonstrated a dose-dependent TYR-I activity. At 22 and 44 μM, piperine significantly reduced the TYR activity by 11.18 ± 2.42 % and 21.51 ± 2.00 %, respectively, compared to the control group. Kojic acid, used as a positive control, significantly inhibited the cellular TYR activity by 68.94 ± 3.57 % at a concentration of 1407 μM. This inhibition coincided with a decrease in the brown color of the solution, confirming a decreased dopachrome formation.

3.4. Effect of piperine on melanin content

The inhibitory effect of piperine on melanin production was investigated using both intracellular and extracellular melanin content assays. For this, B16F10 cells were treated with various concentrations of piperine (11, 22, and 44 μM) and kojic acid (1407 μM) for 72 h in the presence of 10 nM α-MSH. Melanin content in all experiments was calculated from a standard curve of synthetic melanin ($y = 0.0062x + 0.0461$; $R^2 = 1$) and compared against untreated cells. As depicted in Fig. 5A, when B16F10 cells were treated with increasing concentrations of piperine, the intracellular melanin content was significantly reduced by 30.75 ± 1.68, 29.98 ± 1.08, and 37.52 ± 2.53 % at 11, 22, and 44 μM piperine, respectively. This reduction coincided with a decrease in the brown color of the

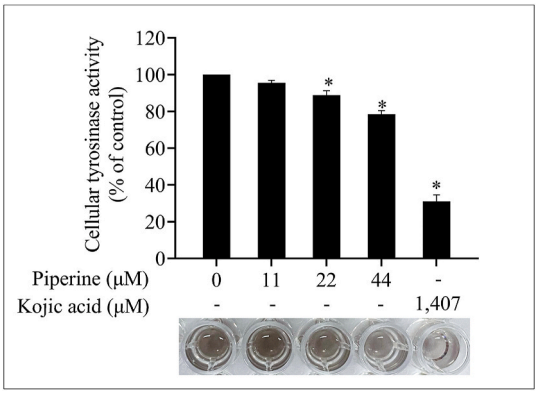


Fig. 4. Effect of piperine and kojic acid treatment for 48 h on the cellular TYR activity of B16F10 melanoma cells. Data are shown as the mean ± S.D. from three independent experiments. * Indicates significant differences from the control group [$p < 0.05$; one-way ANOVA and PostHoc (Tukey) test]. The bottom panel represents the color intensity of dopachrome production.

solution, indicating a decreased melanin production inside the cells. The extracellular melanin was also measured (Fig. 5B), but the results were inconsistent with the intracellular melanin content. That is, increasing concentrations of piperine did not reduce the extracellular melanin content. These results coincided with the color of the secreted melanin in the CM of the piperine-treated group, which did not differ from the control group. In contrast, kojic acid significantly reduced the intracellular and extracellular melanin content by 42.54 ± 1.62 % and 43.96 ± 0.73 %, respectively, resulting in the faded color intensity (Fig. 5A and B).

3.5. *Effect of piperine on the transcriptional expression of genes involved in melanogenesis*

The relative changes in the expression of three melanogenesis-related genes –*TYR*, *TRP-1*, and *TRP-2* – were investigated by RT-qPCR. B16F10 cells were treated with various concentrations of piperine (11, 22, and 44 μM) and kojic acid (1407 μM) for 48 h in the presence of α-MSH. After treatment, total RNA was isolated from the cell lysate and subjected to one-step RT-qPCR using gene fragment-specific primers with *GAPDH* as the internal reference control. As shown in Fig. 6, the expression levels of the *TYR* gene transcript were significantly decreased by 25.6 ± 9.24 % after treatment with piperine at 44 μM compared to the control cells (Fig. 6A). The *TRP-1* gene was significantly down-regulated by 24.68 ± 9.47 and 29.64 ± 6.29 % after treatment with piperine at 22 and 44 μM, respectively, compared to the control (Fig. 6B). However, the expression level of the *TRP-2* gene did not show a significant change after treatment with piperine (Fig. 6C). Thus, piperine inhibits *TYR* and *TRP-1* at the transcriptional level in B16F10 cells. The positive control, kojic acid at 1407 μM, significantly decreased the expression levels of *TYR*, *TRP-1*, and *TRP-2* genes by 49.62 ± 3.47, 31.57 ± 6.77, and 28.10 ± 13.12 %, respectively. Therefore, kojic acid reduces melanin synthesis in B16F10 cells by down-regulating the expression of *TYR*, *TRP-1*, and *TRP-2* genes.

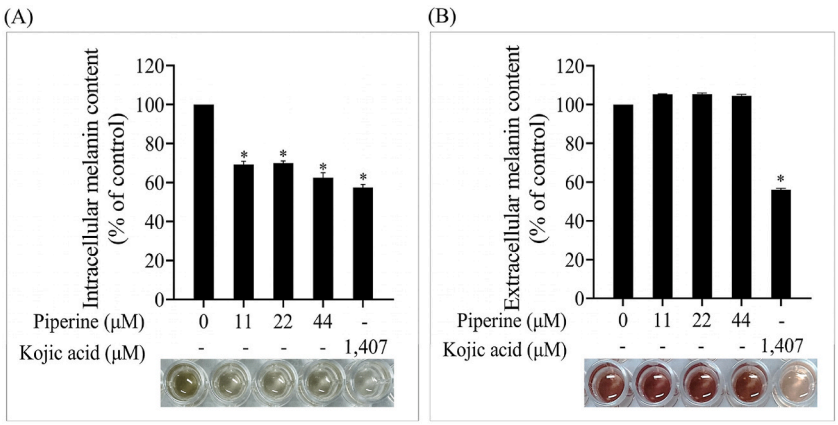


Fig. 5. Effect of 72-h treatment with piperine and kojic acid on the (A) intracellular and (B) extracellular melanin content in B16F10 melanoma cells. Melanin content is reported relative to the control group (set at 100 %). Data are presented as the mean ± S.D. from three independent experiments. * Indicates significant differences from the control group [($p < 0.05$; one-way ANOVA and PostHoc (Tukey) test]. The bottom panel illustrates the color intensity of (A) dissolved melanin after cell lysis and (B) melanin secreted into the CM.

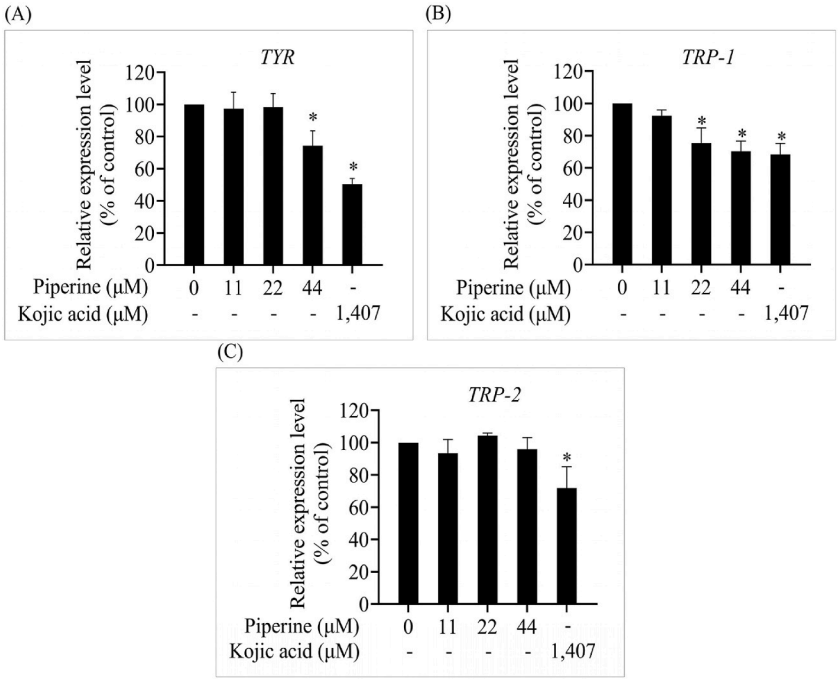


Fig. 6. Effects of piperine (11, 22, and 44 μM) and kojic acid (1407 μM) treatment for 48 h on the relative changes in the transcript level of genes involved in melanogenesis in B16F10 cells. The *GAPDH*-normalized mRNA expression of (A) *TYR*, (B) *TRP-1*, and (C) *TRP-2* relative to the untreated group. Results are presented as the mean ± SD of three independent experiments. * Indicates significant differences from the control group [$p < 0.05$; one-way ANOVA and PostHoc (Tukey) test].

4. Discussion

Melanin is the pigment produced in the melanosomes of melanocytes and plays a crucial role in absorbing UVR to prevent DNA damage. Additionally, it acts as a scavenger of reactive oxygen species to protect cells from oxidative stress [2,36,37], which is the cause of aging, diabetes, and cancer [38]. Although melanin provides protective properties to skin cells, overproduction of melanin after prolonged exposure to UVR and inflammation leads to hyperpigmentation diseases, including melasma, freckles, and age spots [6]. Therefore, seeking natural anti-melanogenesis agents with a TYR-I activity or properties that suppress melanogenesis-related genes without side effects to relieve hyperpigmentation is essential.

In this work, ten compounds purified from different plant sources were screened for their *in vitro* TYR-I activity. Kojic acid was chosen as the positive control because it effectively inhibits both monophenolase and diphenolase activities of mushroom TYR. Additionally, it diminishes intercellular melanin content by inhibiting B16F10 murine TYR activity [39]. A mushroom TYR was used as the assay model due to its easy accessibility and its binuclear copper cluster, similar to human tyrosinase [40]. Among the ten selected plant compounds, piperine derived from *Piper nigrum* exhibited the highest mushroom TYR-I activity. However, its TYR-I activity was over 2.4-fold lower than that of kojic acid at the same concentration. This is in accord with a previous study that reported that piperine inhibits mushroom TYR by 36.1 % at a concentration of 1 mg/mL [41]. Safithri et al. stated that piperine binds to mushroom TYR by forming one hydrogen bond using its aromatic oxygen atom and three hydrophobic interactions with its alkenyl chain. In comparison, kojic acid binds more tightly by forming three hydrogen bonds and two hydrophobic interactions, resulting in its higher TYR-I activity [42]. In this study, pinocembrin, a flavanone, showed a low TYR-I activity. Although this was slightly lower than that in a previous report [43], the concentration of pinocembrin in our study was two times lower. Whilst the chemical structure of pinocembrin contains two –OH groups on ring A, it lacks the –OH group at ring B that plays an important role in the TYR-I activity of flavanone compounds by chelating with the Cu^{2+} atoms in the TYR active site [14]. Alpinetin, a flavanone with only one –OH group that is directly connected to the ring A, also showed a low TYR-I property. The number and position of –OH groups on the chalcone structure influences the TYR-I activity [34]. Thus, chalcone with two –OH groups at positions 2 and 4 on both rings A and B showed the strongest TYR inhibition, whereas cardamomin, a chalcone with only two –OH groups at positions 4 and 6 on ring A, showed a weak TYR-I activity [44]. Likewise, malabaricone A showed no TYR-I activity, whereas malabaricone C exhibited TYR-I activity [45]. This difference may be attributed to the two additional –OH groups on another aromatic ring that play an important role in TYR binding. Although samin and sesamin share a similar aromatic moiety as piperine, their TYR-I activity was much lower than that of piperine. These results are consistent with a previous study that found that sesamin, with a bulky structure like sesamolin, exhibited only a slight TYR-I activity, whereas sesamol, which has a smaller molecular structure, showed an excellent TYR-I activity [17]. Thus, smaller phenolic compounds may have a better TYR binding ability and so induce a higher TYR-I activity. Lansioside compounds, classified as triterpene glycosides,

did not exhibit a good TYR-I property, even though some triterpenes, like poricoic acid A, have been shown to have a strong TYR-I effect against both mushroom and B16 TYR [46]. Moreover, askendoside B, a triterpenoid glycoside, showed a comparable TYR-I activity to that of kojic acid [47]. The difference in the chemical structure of our triterpenoids compared to those reported is the absence of an –OH group on a cyclic structure, which affects the TYR-I activity of terpenoid compounds. Fortunately, piperine is one of the ten selected compounds with the potential to inhibit mushroom TYR. This could be attributed to its close chemical structure similarity to L-DOPA, which is a substrate for tyrosinase.

According to the *in vitro* mushroom TYR assay for screening for TYR-I activity, piperine exhibited the highest TYR-I activity of the ten tested compounds, leading us to study its cellular TYR inhibition and anti-melanogenesis activity in B16F10 mouse melanoma cells. These cells are commonly used as models for anti-melanogenesis studies due to their melanin production ability, α-MSH responsivity, ease of cultivation *in vitro*, and a similar melanogenesis mechanism as human melanocyte [48,49]. The cytotoxicity of piperine on B16F10 cells was examined first to ensure that the anti-melanogenesis effect was not achieved by reducing viable cell numbers. We found that the maximum non-cytotoxic concentration (relative cell viability above 80 % [50]) of piperine was 44 μM. The IC_{50} of piperine on B16F10 cells was 90.16 ± 4.497 μM, consistent with a previous report for piperine on B16 cells of 69.9 μM [51], and so supporting the reliability of the B16F10 cell line and MTT assay procedure. Compared to kojic acid, piperine exhibited higher cytotoxicity to B16F10 cells, which are classified as cancer cells. This result is consistent with previous reports indicating that piperine showed anticancer properties by being cytotoxic to diverse cancer cell lines [52,53], suggesting its potential utility in treating malignancies lacking targeted therapies, such as anaplastic thyroid carcinoma, when complemented with standard cancer medications [54,55]. At the cellular level, piperine induced a dose-dependent TYR-I activity with a much lower required concentration to inhibit cellular TYR activity than in the *in vitro* mushroom TYR study, indicating a more effective inhibition by piperine on TYR in B16F10 cells compared to mushroom TYR. This is consistent with a previous study which revealed that the *Sargassum polycystum* ethanolic extract showed only a slight TYR-I activity against mushroom TYR but a higher TYR-I activity against B16F10 cellular TYR [56]. These authors suggested that using the B16F10 cellular TYR, a membrane-bound enzyme, is more reliable for screening for TYR-I activity than the mushroom TYR, a cytosol enzyme [56]. Moreover, the difference in the number of cysteine residues responsible for protein stabilization via disulfide bonds results in the difference in the accessibility of TYR-Is to the active site of mushroom and mouse TYR [57].

For the intracellular melanin content, 44 μM piperine showed the interesting property of diminishing the intracellular melanin production more than kojic acid at 1407 μM, indicating a lower effective concentration for piperine than kojic acid at the cellular level. However, in our study piperine did not influence the extracellular melanin content. In accordance, 35 μM piperine was previously shown to reduce the intracellular melanin level by 39 %, comparable to that with 70 μM kojic acid, whereas the extracellular melanin content did not decrease [58]. At the cellular level, factors influencing extracellular melanin content include not only melanin production levels but also melanosome transporting ability [59]. The inhibition of these processes results in the reduction of extracellular melanin content. There have been reports that some depigmenting agents act through melanin transport inhibition. For example, 16-kauren and 2-methyl-naphtho [1,2,3-de] quinolin-8-one have been reported as anti-melanogenesis agents by downregulating melanin transport genes, including *MLPH*, *Rab27a*, and *MYO5a* genes [60,61]. Therefore, piperine might not suppress the melanosome transport process, leading to the accumulation of extracellular melanin. To the best of our knowledge, the effect of piperine on melanogenesis-related gene expression is unknown. Here, we showed that piperine can suppress expression of *TYR* and *TRP-1* but not *TRP-2* gene transcripts. A similar feature has also been found in some previous reports. For example, decursin attenuated melanogenesis by suppressing only *TYR* and *TRP-1* but not *TRP-2* transcript expression levels in B16F10 cells [62], while sargahydroquinoic acid also suppressed only these two genes, leading to the lowering of both intracellular and extracellular melanin content [63]. Although piperine could not diminish extracellular melanin content, it effectively reduced total melanin production in B16F10 cells by downregulating *TYR* and *TRP-1* transcript expression levels and by the direct inhibition of cellular TYR. Furthermore, it has been previously reported that piperine possesses anti-metastatic properties in B16F10 cells by inactivating cAMP-response element binding protein (CREB) [64]. CREB is a transcription factor that controls various cellular responses, including proliferation, survival, and differentiation [65]. Besides being an inflammatory regulator, CREB also induces the expression of MITF, the master regulator of melanogenesis, which in turn regulates the expression of *TYR*, *TRP-1*, and *TRP-2* [5]. Thus, the observed reduction in melanin in this study may be attributed to the CREB inactivation by piperine. To elucidate the underlying anti-melanogenesis mechanism of piperine, a greater number of genes directly involved in melanin synthesis or the knockdown of unrelated genes, are conducted in further study.

5. Conclusions

Various secondary metabolites derived from plants can potentially function as TYR-Is. However, their chemical structures should be accessible and bind properly with the TYR active site. Among the ten plant compounds selected for study, piperine from *Piper nigrum* exhibited the strongest *in vitro* TYR-I activity. At the cellular level, although piperine is more cytotoxic to B16F10 melanoma cells than kojic acid, it is not cytotoxic at lower but effective concentrations where piperine can significantly decrease melanin production by directly inhibiting TYR and suppressing transcription of the *TYR* and *TRP-1* genes. The findings demonstrate that piperine holds promise as a potential agent for reducing melanin production. However, a greater number of genes involved in melanin synthesis and melanin transport should be further studied in order to elucidate the molecular mechanisms underlying piperine's anti-melanogenic effects. Also, the *in vivo* study should be investigated. Moreover, structural derivatization of piperine should be studied to enhance its anti-melanogenic properties for development as an alternative additive in cosmetics and pharmaceutical products aimed at treating hyperpigmentation.

Ethics

This work did not involve with ethical considerations or approval.

Data accessibility

Data will be made available on request.

CRediT authorship contribution statement

Phanthiwa Khongkarat: Writing – original draft, Resources, Methodology, Formal analysis. **Ponglada Sadangrit:** Resources, Methodology, Formal analysis. **Songchan Puthong:** Methodology. **Thitipan Meemongkolkiat:** Methodology. **Preecha Phuwapraisirisan:** Writing – review & editing, Supervision, Investigation. **Chanpen Chanchao:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Ratchadaphiseksomphot Endowment Fund for Postdoctoral Fellowship (Chulalongkorn University), the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), the Plant Genetic Conservation Project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn - Chulalongkorn University, and the National Science and Technology Development Agency.

References

[1] H. Ando, Y. Niki, M. Ito, K. Akiyama, M.S. Matsui, D.B. Yarosh, M. Ichihashi, Melanosomes are transferred from melanocytes to keratinocytes through the processes of packaging, release, uptake, and dispersion, J. Invest. Dermatol. 132 (4) (2012) 1222–1229, https://doi.org/10.1038/jid.2011.413.
[2] G.-E. Costin, V.J. Hearing, Human skin pigmentation: melanocytes modulate skin color in response to stress, Faseb. J. 21 (4) (2007) 976–994, https://doi.org/10.1096/fj.06-6649rev.
[3] S.A. D'Mello, G.J. Finlay, B.C. Baguley, M.E. Askarian-Amiri, Signaling pathways in melanogenesis, Int. J. Mol. Sci. 17 (7) (2016) 1144, https://doi.org/10.3390/ijms17071144.
[4] Y. Hushcha, I. Blo, L. Oton-Gonzalez, G.D. Mauro, F. Martini, M. Tognon, M.D. Mattei, microRNAs in the regulation of melanogenesis, Int. J. Mol. Sci. 22 (11) (2021) 6104, https://doi.org/10.3390/ijms22116104.
[5] J. Vachtenheim, J. Borovanský, "Transcription physiology" of pigment formation in melanocytes: central role of MITF, Exp. Dermatol. 19 (7) (2010) 617–627, https://doi.org/10.1111/j.1600-0625.2009.01053.x.
[6] T. Pillaiyar, V. Namasivayam, M. Manickam, S.-H. Jung, Inhibitors of melanogenesis: an updated review, J. Med. Chem. 61 (17) (2018) 7395–7418, https://doi.org/10.1021/acs.jmedchem.7b00967.
[7] L. Liu, G. Song, Z. Song, Intrinsic atopic dermatitis and extrinsic atopic dermatitis: similarities and differences, Clin. Cosmet. Invest. Dermatol. (2022) 2621–2628, https://doi.org/10.2147/CCID.S391360.
[8] N. Silpa-Archa, I. Kohli, S. Chaowattanapanit, H.W. Lim, I. Hamzavi, Postinflammatory hyperpigmentation: a comprehensive overview: Epidemiology, pathogenesis, clinical presentation, and noninvasive assessment technique, J. Am. Acad. Dermatol. 77 (4) (2017) 591–605, https://doi.org/10.1016/j.jaad.2017.01.035.
[9] T. Panichakul, T. Rodboon, P. Suwannalert, C. Tripetch, R. Rungruang, N. Boohuad, P. Youdee, Additive effect of a combination of *Artocarpus lakoocha* and *Glycyrrhiza glabra* extracts on tyrosinase inhibition in melanoma B16 cells, Pharmaceuticals 13 (10) (2020) 310, https://doi.org/10.3390/ph13100310.
[10] M. Saeedi, M. Eslamifar, K. Khezri, Kojic acid applications in cosmetic and pharmaceutical preparations, Biomed. Pharmacother. 110 (2019) 582–593, https://doi.org/10.1016/j.biopha.2018.12.006.
[11] E.V. Curto, C. Kwong, H. Hermersdörfer, H. Glatt, C. Santis, V. Virador, V.J. Hearing Jr., T.P. Dooley, Inhibitors of mammalian melanocyte tyrosinase: *in vitro* comparisons of alkyl esters of gentisic acid with other putative inhibitors, Biochem. Pharmacol. 57 (6) (1999) 663–672, https://doi.org/10.1016/S0006-2952(98)00340-2.
[12] W. Westerhof, T. Kooyers, Hydroquinone and its analogues in dermatology–a potential health risk, J. Cosmet. Dermatol. 4 (2) (2005) 55–59, https://doi.org/10.1111/j.1473-2165.2005.40202.x.
[13] J. Gubitosa, V. Rizzi, P. Fini, R. Del Sole, A. Lopedota, V. Laquintana, N. Denora, A. Agostiano, P. Cosma, Multifunctional green synthetized gold nanoparticles/chitosan/ellagic acid self-assembly: antioxidant, sun filter and tyrosinase-inhibitor properties, Mater. Sci. Eng. C 106 (2020) 110170, https://doi.org/10.1016/j.msec.2019.110170.
[14] R.J. Obaid, E.U. Mughal, N. Naeem, A. Sadiq, R.I. Alsantali, R.S. Jassas, Z. Moussa, S.A. Ahmed, Natural and synthetic flavonoid derivatives as new potential tyrosinase inhibitors: a systematic review, RSC Adv. 11 (36) (2021) 22159–22198, https://doi.org/10.1039/D1RA03196A.
[15] Y.-M. Won, Z.-K. Seong, J.-L. Kim, H.-S. Kim, H.-H. Song, D.-Y. Kim, J.-H. Kim, S.-R. Oh, H.-W. Cho, J.-H. Cho, Triterpene glycosides with stimulatory activity on melanogenesis from the aerial parts of *Weigela subsessilis*, Arch Pharm. Res. (Seoul) 38 (2015) 1541–1551, https://doi.org/10.1007/s12272-014-0524-0.
[16] C.L. Cespedes, C. Balbontin, J.G. Avila, M. Dominguez, J. Alarcon, C. Paz, V. Burgos, L. Ortiz, I. Peñaloza-Castro, D.S. Seigler, Inhibition on cholinesterase and tyrosinase by alkaloids and phenolics from *Aristotelia chilensis* leaves, Food Chem. Toxicol. 109 (2017) 984–995, https://doi.org/10.1016/j.fct.2017.05.009.
[17] M. Srisayam, N. Weerapreeyakul, K. Kanokmedhakul, Inhibition of two stages of melanin synthesis by sesamol, sesamin and sesamolin, Asian Pac. J. Trop. Biomed. 7 (10) (2017) 886–895, https://doi.org/10.1016/j.apjtb.2017.09.013.
[18] H.-D. Kim, H. Choi, F. Abekura, J.-Y. Park, W.-S. Yang, S.-H. Yang, C.-H. Kim, Naturally-occurring tyrosinase inhibitors classified by enzyme kinetics and copper chelation, Int. J. Mol. Sci. 24 (9) (2023) 8226, https://doi.org/10.3390/ijms24098226.
[19] N.Z. Mamadalieva, F.S. Youssef, H. Hussain, G. Zengin, A. Mollica, N.M. Al Musayeib, M.L. Ashour, B. Westermann, L.A. Wessjohann, Validation of the antioxidant and enzyme inhibitory potential of selected triterpenes using *in vitro* and *in silico* studies, and the evaluation of their ADMET properties, Molecules 26 (21) (2021) 6331, https://doi.org/10.3390/molecules26216331.

[20] G. Zhao, Y. Tong, F. Luan, W. Zhu, C. Zhan, T. Qin, W. An, N. Zeng, A review of its pharmacology and pharmacokinetics, Front. Pharmacol. 13 (2022) 814370, https://doi.org/10.3389/fphar.2022.814370. DOI: 10.5772/intechopen.108030Alpinetin.

[21] L.M. Goncalves, I.M. Valente, J.A. Rodrigues, An overview on cardamonin, J. Med. Food 17 (6) (2014) 633–640, https://doi.org/10.1089/jmf.2013.0061.

[22] H.M. Abdallah, G.A. Mohamed, S.R. Ibrahim, *Lansium domesticum*—a fruit with multi-benefits: traditional uses, phytochemicals, nutritional value, and bioactivities, Nutrients 14 (7) (2022) 1531, https://doi.org/10.3390/nu14071531.

[23] R. Ramadhan, W. Worawalai, P. Phuwapraisirisan, New onoceranoid xyloside from *Lansium parasiticum*, Nat. Prod. Res. 33 (20) (2019) 2917–2924, https://doi.org/10.1080/14786419.2018.1510395.

[24] R. Ramadhan, P. Phuwapraisirisan, Arylalkanones from *Horsfieldia macrobotrys* are effective antidiabetic agents achieved by α-glucosidase inhibition and radical scavenging, Nat. Prod. Commun. 10 (2) (2015), https://doi.org/10.1177/1934578X1501000230, 1934578X1501000230.

[25] I.U. Haq, M. Imran, M. Nadeem, T. Tufail, T.A. Gondal, M.S. Mubarak, Piperine: a review of its biological effects, Phytother Res. 35 (2) (2021) 680–700, https://doi.org/10.1002/ptr.6855.

[26] M.H. Elbatreek, I. Mahdi, W. Ouchari, M.F. Mahmoud, M. Sobeh, Current advances on the therapeutic potential of pinocembrin: an updated review, Biomed. Pharmacother. 157 (2023) 114032, https://doi.org/10.1016/j.biopha.2022.114032.

[27] W. Worawalai, P. Phuwapraisirisan, Samin-derived flavonolignans, a new series of antidiabetic agents having dual inhibition against α-glucosidase and free radicals, Nat. Prod. Res. 34 (22) (2020) 3169–3175, https://doi.org/10.1080/14786419.2018.1553169.

[28] E.M. Henriques Monteiro, L.A. Chibli, C.H. Yamamoto, M.C. Santana Pereira, F.M. Pinto Vilela, M.P. Rodarte, M.A.d. Oliveira Pinto, M.d.P. Henriques do Amaral, M. Silva Silvério, A.L.S.d. Matos Araújo, Antinociceptive and anti-inflammatory activities of the sesame oil and sesamin, Nutrients 6 (5) (2014) 1931–1944, https://doi.org/10.3390/nu6051931.

[29] P. Sadangrit, S. Puthong, R. Ramadhan, P. Phuwapraisirisan, C. Chanchao, Reduction of melanin content and tyrosinase and tyrosinase-related protein gene expression by horsfieldone A and maingayone D, Curr. Top. 18 (2022) 29–43. http://www.researchtrends.net/tia/article_pdf.asp?in=0&vn=18&tid=50&aid=6982.

[30] J. Cao, C. Chen, Y. Wang, X. Chen, Z. Chen, X. Luo, Influence of autologous dendritic cells on cytokine-induced killer cell proliferation, cell phenotype and antitumor activity *in vitro*, Oncol. Lett. 12 (3) (2016) 2033–2037, https://doi.org/10.3892/ol.2016.4839.

[31] E. Ketata, H. Elleuch, A. Neifar, W. Mihoubi, W. Ayadi, N. Marrakchi, F. Rezgui, A. Gargouri, Anti-melanogenesis potential of a new series of Morita-Baylis-Hillman adducts in B16F10 melanoma cell line, Bioorg. Chem. 84 (2019) 17–23, https://doi.org/10.1016/j.bioorg.2018.11.028.

[32] M. Chatatikun, T. Yamauchi, K. Yamasaki, S. Aiba, A. Chiabchalard, Anti melanogenic effect of *Croton roxburghii* and *Croton sublyratus* leaves in α-MSH stimulated B16F10 cells, J. Tradit. Complement. Med. 9 (1) (2019) 66–72, https://doi.org/10.1016/j.jtcme.2017.12.002.

[33] Z.E. Jiménez-Pérez, P. Singh, Y.-J. Kim, R. Mathiyalagan, D.-H. Kim, M.H. Lee, D.C. Yang, Applications of P*anax ginseng* leaves-mediated gold nanoparticles in cosmetics relation to antioxidant, moisture retention, and whitening effect on B16BL6 cells, J. Ginseng Res. 42 (3) (2018) 327–333, https://doi.org/10.1016/j.jgr.2017.04.003.

[34] K. Kim, A.S. Leutou, H. Jeong, D. Kim, C.N. Seong, S.-J. Nam, K.-M. Lim, Anti-pigmentary effect of (-)-4-hydroxysattabacin from the marine-derived bacterium *Bacillus* sp, Mar. Drugs 15 (5) (2017) 138, https://doi.org/10.3390/md15050138.

[35] H. Xu, L. Li, S. Wang, Z. Wang, L. Qu, C. Wang, K. Xu, Royal jelly acid suppresses hepatocellular carcinoma tumorigenicity by inhibiting H3 histone lactylation at H3K9la and H3K14la sites, Phytomedicine 118 (2023) 154940, https://doi.org/10.1016/j.phymed.2023.154940.

[36] J.-J. Oh, J.Y. Kim, S.H. Son, W.-J. Jung, J.-W. Seo, G.-H. Kim, Fungal melanin as a biocompatible broad-spectrum sunscreen with high antioxidant activity, RSC Adv. 11 (32) (2021) 19682–19689, https://doi.org/10.1039/d1ra02583j.

[37] X. Fu, M. Xie, M. Lu, L. Shi, T. Shi, M. Yu, Characterization of the physicochemical properties, antioxidant activity, and antiproliferative activity of natural melanin from *S. reiliana*, Sci. Rep. 12 (1) (2022) 2110, https://doi.org/10.1038/s41598-022-05676-z.

[38] P.D. Ray, B.-W. Huang, Y. Tsuji, Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling, Cell. Signal. 24 (5) (2012) 981–990, https://doi.org/10.1016/j.cellsig.2012.01.008.

[39] W. Wang, Y. Gao, W. Wang, J. Zhang, J. Yin, T. Le, J. Xue, U.H. Engelhardt, H. Jiang, Kojic acid showed consistent inhibitory activity on tyrosinase from mushroom and in cultured B16F10 cells compared with arbutins, Antioxidants 11 (3) (2022), https://doi.org/10.3390/antiox11030502.

[40] S.-Y. Seo, V.K. Sharma, N. Sharma, Mushroom tyrosinase: recent prospects, J. Agric. Food Chem. 51 (10) (2003) 2837–2853, https://doi.org/10.1021/jf020826f.

[41] T. Yodsawad, T. Meemongkolkiat, C. Chanchao, T. Damsud, P. Phuwapraisirisan, Four new alkylamides from the fruits of *Piper retrofractum* and antityrosinase evaluation, Nat. Prod. Res. (2023) 1–10, https://doi.org/10.1080/14786419.2023.2258542.

[42] M. Safithri, D. Andrianto, A.G. Arda, P.H. Syaifie, N.M.N. Kaswati, E. Mardliyati, D. Ramadhan, M.M. Jauhar, D.W. Nugroho, D.A. Septaningsih, The effect of red betel (*Piper crocatum*) water fraction as tyrosinase inhibitors: *in vitro*, molecular docking, and molecular dynamics studies, J. King Saud Univ. Sci. 35 (10) (2023) 102933, https://doi.org/10.1016/j.jksus.2023.102933.

[43] M.B. Mapunya, A.A. Hussein, B. Rodriguez, N. Lall, Tyrosinase activity of *Greyia flanaganii* (Bolus) constituents, Phytomedicine 18 (11) (2011) 1006–1012, https://doi.org/10.1016/j.phymed.2011.03.013.

[44] S. Khatib, O. Nerya, R. Musa, M. Shmuel, S. Tamir, J. Vaya, Chalcones as potent tyrosinase inhibitors: the importance of a 2, 4-substituted resorcinol moiety, Biorg. Med. Chem. 13 (2) (2005) 433–441, https://doi.org/10.1016/j.bmc.2004.10.010.

[45] H. Gao, Predicting tyrosinase inhibition by 3D QSAR pharmacophore models and designing potential tyrosinase inhibitors from Traditional Chinese medicine database, Phytomedicine 38 (2018) 145–157, https://doi.org/10.1016/j.phymed.2017.11.012.

[46] S.-h. Hu, G. Zhou, Y.-w. Wang, Tyrosinase inhibitory activity of total triterpenes and poricoic acid A isolated from *Poria cocos*, Chin. Herb. Med. 9 (4) (2017) 321–327, https://doi.org/10.1016/S1674-6384(17)60111-4.

[47] M.T.H. Khan, M.I. Choudhary, R.P. Mamedova, M.A. Agzamova, M.N. Sultankhodzhaev, M.I. Isaev, Tyrosinase inhibition studies of cycloartane and cucurbitane glycosides and their structure–activity relationships, Biorg. Med. Chem. 14 (17) (2006) 6085–6088, https://doi.org/10.1016/j.bmc.2006.05.002.

[48] R. Busca, R. Ballotti, Cyclic AMP a key messenger in the regulation of skin pigmentation, Pigm. Cell Res. 13 (2) (2000) 60–69, https://doi.org/10.1034/j.1600-0749.2000.130203.x.

[49] S.M. An, H.J. Kim, J.E. Kim, Y.C. Boo, Flavonoids, taxifolin and luteolin attenuate cellular melanogenesis despite increasing tyrosinase protein levels, Phytother Res. 22 (9) (2008) 1200–1207, https://doi.org/10.1002/ptr.2435.

[50] B. Guo, Y. Sun, A. Finne-Wistrand, K. Mustafa, A.-C. Albertsson, Electroactive porous tubular scaffolds with degradability and non-cytotoxicity for neural tissue regeneration, Acta Biomater. 8 (1) (2012) 144–153, https://doi.org/10.1016/j.actbio.2011.09.027.

[51] D.P. Bezerra, C. Pessoa, M.O.d. Moraes, E.R. Silveira, M.A.S. Lima, F.J. Martins Elmiro, L.V. Costa-Lotufo, Antiproliferative effects of two amides, piperine and piplartine, from *Piper* species, Z. Naturforsch. C Biosci. 60 (7–8) (2005) 539–543, https://doi.org/10.1515/znc-2005-7-805.

[52] A. Fattah, A. Morovati, Z. Niknam, L. Mashouri, A. Asadi, S.T. Rizi, M. Abbasi, F. Shakeri, O. Abazari, The synergistic combination of cisplatin and piperine induces apoptosis in MCF-7 cell line, Iran. J. Public Health 50 (5) (2021) 1037, https://doi.org/10.18502/ijph.v50i5.6121.

[53] L. Guo, Y. Yang, Y. Sheng, J. Wang, S. Ruan, C. Han, Mechanism of piperine in affecting apoptosis and proliferation of gastric cancer cells via ROS-mitochondria-associated signalling pathway, J. Cell Mol. Med. 25 (20) (2021) 9513–9522, https://doi.org/10.1111/jcmm.16891.

[54] Y. Liu, Y. Zhang, Z. Tan, J. Wang, Y. Hu, J. Sun, M. Bao, P. Huang, M. Ge, Y.J. Chai, Lysyl oxidase promotes anaplastic thyroid carcinoma cell proliferation and metastasis mediated via BMP1, Gland Surg. 11 (1) (2022) 245, https://doi.org/10.21037/gs-21-908.

[55] C.R. Quijia, M. Chorilli, Piperine for treating breast cancer: a review of molecular mechanisms, combination with anticancer drugs, and nanosystems, Phytother Res. 36 (1) (2022) 147–163, https://doi.org/10.1002/ptr.7291.

[56] Y. Chan, K. Kim, S. Cheah, Inhibitory effects of *Sargassum polycystum* on tyrosinase activity and melanin formation in B16F10 murine melanoma cells, J. Ethnopharmacol. 137 (3) (2011) 1183–1188, https://doi.org/10.1016/j.jep.2011.07.050.

[57] C.W. Van Gelder, W.H. Flurkey, H.J. Wichers, Sequence and structural features of plant and fungal tyrosinases, Phytochemistry (Elsevier) 45 (7) (1997) 1309–1323, https://doi.org/10.1016/S0031-9422(97)00186-6.

[58] S.V. Luca, K. Gaweł-Bęben, M. Strzępek-Gomółka, K. Czech, A. Trifan, G. Zengin, I. Korona-Glowniak, M. Minceva, J. Gertsch, K. Skalicka-Woźniak, Insights into the phytochemical and multifunctional biological profile of spices from the genus *Piper*, Antioxidants 10 (10) (2021) 1642, https://doi.org/10.3390/antiox10101642.
[59] K. Nagashima, S. Torii, Z. Yi, M. Igarashi, K. Okamoto, T. Takeuchi, T. Izumi, Melanophilin directly links Rab27a and myosin Va through its distinct coiled-coil regions, FEBS Lett. 517 (1–3) (2002) 233–238, https://doi.org/10.1016/S0014-5793(02)02634-0.
[60] J.I. Park, H.Y. Lee, J.E. Lee, C.H. Myung, J.S. Hwang, Inhibitory effect of 2-methyl-naphtho [1, 2, 3-de] quinolin-8-one on melanosome transport and skin pigmentation, Sci. Rep. 6 (1) (2016) 29189, https://doi.org/10.1038/srep29189.
[61] C.H. Myung, K. Kim, J. il Park, J.E. Lee, J.A. Lee, S.C. Hong, K.-M. Lim, J.S. Hwang, 16-Kauren-2-beta-18, 19-triol inhibits melanosome transport in melanocytes by down-regulation of melanophilin expression, J. Dermatol. Sci. 97 (2) (2020) 101–108, https://doi.org/10.1016/j.jdermsci.2019.12.009.
[62] H. Choi, J.-H. Yoon, K. Youn, M. Jun, Decursin prevents melanogenesis by suppressing MITF expression through the regulation of PKA/CREB, MAPKs, and PI3K/Akt/GSK-3β cascades, Biomed. Pharmacother. 147 (2022) 112651, https://doi.org/10.1016/j.biopha.2022.112651.
[63] M.S. Azam, J.-I. Kim, C.G. Choi, J. Choi, B. Lee, H.-R. Kim, Sargahydroquinoic acid suppresses hyperpigmentation by cAMP and ERK1/2-mediated downregulation of MITF in α-MSH-stimulated B16F10 cells, Foods 10 (10) (2021) 2254, https://doi.org/10.3390/foods10102254.
[64] C. Pradeep, G. Kuttan, Piperine is a potent inhibitor of nuclear factor-κB (NF-κB), c-Fos, CREB, ATF-2 and proinflammatory cytokine gene expression in B16F-10 melanoma cells, Int. Immunopharm. 4 (14) (2004) 1795–1803, https://doi.org/10.1016/j.intimp.2004.08.005.
[65] A.Y. Wen, K.M. Sakamoto, L.S. Miller, The role of the transcription factor CREB in immune function, J. Immunol. 185 (11) (2010) 6413–6419, https://doi.org/10.4049/jimmunol.1001829.