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Original Article

Reduction of melanin content and tyrosinase and tyrosinase-related protein gene expression by horsfieldone A and maingayone D

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ABSTRACT

Horsfieldone A and maingayone D purified from *Horsfieldia motley*, a native plant in Indonesia, were screened in this work. To determine the potential effects of these plant compounds on melanogenesis, their tyrosinase inhibitor (TYR-I) activity was evaluated using *in vitro* and cellular screening. Melanin content was measured in B16F10 melanoma cells. Altered transcript expression levels of the TYR, TYR-related protein 1 (TRP-1), and TRP 2 (TRP-2) encoding genes were investigated using quantitative real time reverse transcriptase (qrtRT)-PCR in MSH-treated B16F10 melanoma cells, compared to kojic acid as the positive TYR-I control. Horsfieldone A and maingayone D had an effective TYR-I activity against mushroom TYR with the half maximal inhibitory concentration (IC50 value) of 0.294 and 0.020 mM, respectively, compared with kojic acid (IC50 = 0.048 mM). Considering a relative cell viability of less than 80% at a 72-h treatment as cytotoxic, horsfieldone A and maingayone D exhibited cytotoxicity against B16F10 melanoma cells at concentrations of higher than 0.011 and 0.004 mM, whereas the less cytotoxic kojic acid required 1.407 mM, with IC50 values of 0.021, 0.019, and 10.765 mM for horsfieldone A, maingayone D, and kojic acid, respectively. Kojic acid, but neither horsfieldone A nor maingayone D, exhibited significant TYR-I activity, but all three compounds inhibited melanin production efficiently. The qrtRT-PCR assays showed that horsfieldone A reduced transcript levels of TRP-2, kojic acid reduced TRP-1, and maingayone D reduced both TRP-1 and TRP-2, but none of them reduced TYR transcript levels. Thus, horsfieldone A and maingayone D are potential TYR-Is.

KEYWORDS: B16F10 melanoma cells, cytotoxicity, kojic acid, melanin, tyrosinase.

ABBREVIATIONS

TYR: tyrosinase, TYR-I: tyrosinase inhibitor, TYRP-1: tyrosinase-related protein 1, TYRP-2: tyrosinase-related protein 2, qrtRT-PCR: quantitative real time reverse transcriptase polymerase chain reaction, MSH: α-melanocyte stimulating hormone, IC_{50}: half maximal inhibitory concentration, UV: ultraviolet, ROS: reactive oxygen species, HQ: hydroquinone, DMSO: dimethyl sulfoxide, L-DOPA: L-3,4-dihydroxyphenylalanine, CM: culture medium, DMEM: Dulbecco's modified eagle's medium, EDTA: ethylene diamine tetra acetic acid, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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bromide, PBS: phosphate buffered saline, RT: room temperature, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, SD: standard deviation, ANOVA: one-way analysis of variance, DHICA: 5,6-dihydroxyindole-2-carboxylic acid, MITF: micropthalmia.

INTRODUCTION

Melanin is a group of natural pigments found in various organisms, and provides the dark color to skin, hair, and eyes. Under a normal physiological condition, the primary function of melanin is to protect the skin from ultraviolet (UV) radiation and it may also serve as an antioxidant by scavenging reactive oxygen species (ROS) [1].

Melanin production is controlled by various factors, such as genetics, hormones, local keratinocytes, and Langerhans cells, and is influenced by UV light, inflammation, byproducts from the arachidonic acid cycle, androgens, estrogen, glucocorticoids, and thyroid hormone. Abnormal melanin production can cause pigmentary disorders, such as hypo- and hyper-pigmentation [2-4].

Melanogenesis is a complex pathway involving a series of enzymatic and chemically catalyzed reactions. There are three enzymes that are important in the process of melanin synthesis, namely tyrosinase (TYR), TYR-related protein-1 (TRP-1), and TRP-2 [5]. While these three enzymes are absolutely required for eumelanin synthesis, only TYR is essentially required in pheomelanin synthesis [6, 7].

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) is a glycoprotein located in the melanosomal membrane. This enzyme is widely distributed in nature and is found in high levels in *Aeromonas* sp. SNS (bacteria), *Neurospora crassa* (fungi), and *Agaricus bisporus* (fungi) [8, 9]. The TYR purified from *A. bisporus* champignon is highly homologous with mammalian TYRs [10]. Since TYR plays an essential role in melanogenesis, it has become a widely studied target for melanogenesis inhibitors that directly inhibit TYR catalytic activity. Most commercially available cosmetics or skin whitening agents are TYR inhibitors (TYR-Is). Among different types of compounds that are able to inhibit melanogenesis, such as specific TYR inactivators and TYR-Is, dopaquinone scavengers, alternative enzyme substrates, non-specific enzyme inactivators, and denaturants, it is obvious that only specific TYR inactivators and inhibitors are regarded as true TYR-Is, in that they actually bind to the enzyme and inhibit its activity [10].

In addition to the inhibitory mechanism, the inhibitory strength is the primary standard used for assessing potential TYR-Is. The inhibitory strength is usually expressed as the half maximal inhibitory concentration (IC_{50}) value. For screening new TYR-Is, reference compounds commonly used as positive controls in the TYR inhibition assay are kojic acid, arbutin, and hydroquinone (HQ) [11, 12]. Various known TYR-Is originated from natural, semisynthetic, and synthetic sources. Many TYR-Is, such as HQ, arbutin, kojic acid, azelaic acid, L-ascorbic acid, ellagic acid, and tranexamic acid, have been used as skin whitening agents, but most of them have side effects if an overdose is used. Kojic acid in the range of 1% (w/v) or less is recommended as a safe and efficient cosmetic [13]. However, kojic acid at high doses has some side effects including erythema, stinging sensations, and contact eczema [14, 15]. Therefore, there is a great need to develop new TYR-Is from different sources with high efficacy but less adverse side effects. Plants such as *Lepechinia meyenii*, *Cassia tora*, and *Hypericum laricifolium* Juss. have been one of the most popular alternatives for finding natural TYR-Is [12, 16-19].

Horsfieldone A and maingayone D have been previously purified from *Horsfieldia motleyi* and reported to have antidiabetic and free radical scavenging activities [20] but their TYR-I activity was not addressed. Thus, this study focused on the TYR-I activity of horsfieldone A and maingayone D. For primary screening, the TYR-I activity was evaluated (i) *in vitro* using mushroom TYR and (ii) at a cellular level in B16F10 cells, while the inhibition of melanin production was measured at a cellular level using B16F10 melanoma cells. Furthermore, the possible melanin inhibition mechanism of these two plant compounds was explored by measuring the mRNA expression levels of the *TYR*, *TRP-1*, and *TRP-2* transcripts in α-melanocyte stimulating hormone (MSH)-treated B16F10 melanoma cells. If suitable, the TYR-I compound(s) could be further evaluated for application in cosmetic products.

MATERIALS AND METHODS

Plant compounds

Horsfieldone A and maingayone D, purified from *Horsfieldia motley*, were received from Dr. Preecha Phuwapraisirisan and Dr. Rico Ramadhan. They were dissolved in dimethyl sulfoxide or DMSO (Merck, Germany) and stored at -20 °C as the stock solution.

In vitro mushroom TYR-I activity assay

The TYR-I activity of each compound was examined using the DOPAchrome enzymatic method with L-3,4-dihydroxyphenylalanine or L-DOPA (Abcam, UK) as the substrate, as described previously [21] with slight modifications. The experimental groups were divided into the three groups of control (DMSO), positive control [kojic acid (Merck, Germany)], and the respective treatment (horsfieldone A or maingayone D). The reaction mixture in each well of a 96-well plate was comprised of 120 µL of 1.5 mM L-DOPA in 80 mM phosphate buffer (pH 6.8) and 40 µL of the same buffer or the test compound dissolved in DMSO at various concentrations (0.1, 0.2, and 0.4 mM). The mixture was incubated at 25 °C for 10 min. Then, 40 µL of 165 units/mL mushroom TYR (Merck, Germany) in 50 mM phosphate buffer (pH 6.5) was added and mixed. After 5 min incubation at 25 °C, the absorbance was measured at 475 nm (A_{475}) using a microplate reader (Thermo Fisher Scientific, USA). The data were expressed as the percentage of inhibition of the control TYR activity. Kojic acid was used as a standard TYR-I control. The percentage of TYR-I was calculated as shown in Eq. (1);

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

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

A graph of the percentage TYR-I (on the Y axis) against the concentration of the target compound (on the X axis) was plotted and was used to calculate the IC_{50} value by Probit analysis from triplicate experiments.

Cell culture

B16F10 melanoma cells (American Type Culture Collection, USA) were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. B16F10 melanoma cells (1×10^5 cells/mL) were cultured in 15 mL culture medium [CM; Dulbecco's Modified Eagle's Medium or DMEM (Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin] in a 75-cm^2 cell culture flask at 37 °C in humidified 95% air and 5% CO_2 atmosphere. The cells were subcultured when their confluency reached 80% by removing the CM, and adding 2 mL of 0.1% (w/v) trypsin-EDTA solution (Invitrogen, USA) to the cultured cells for 2 min at 25 ^oC. Then, 1 mL fresh DMEM was added and the detached cells were harvested and washed in CM by centrifugation before being transferred into a new cell culture flask for maintenance. Cells were used at passage 16-29.

Cell viability assay

The relative cell viability was investigated using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. B16F10 melanoma cells were divided into control (cells cultured with DMSO) and treatment (cells cultured with kojic acid, horsfieldone A, or maingayone D) groups. Briefly, B16F10 melanoma cells were seeded at a density of 1×10^4 cells/well/200 µL in 96-well plates and incubated at 37 °C in humidified 95% air and 5% CO_2 atmosphere. After 24 h, the cells were treated with 10 nM α-melanocyte stimulating hormone or α-MSH (Merck, Germany) and incubated for 48 h as before. The cells were then treated with 1% (v/v) DMSO in CM containing various concentrations of the respective compound or without the compound (control) and incubated for 72 h as before. After the incubation period, the CM was removed and replaced with 200 µL fresh CM followed by 10 µL of MTT solution (5 mg/mL MTT in normal saline solution) and incubated as before for 4 h. The media was then removed and 150 µL DMSO was added to dissolve the formazan crystals prior to measuring the absorbance at 540 nm (A_{540}) using a microplate reader. The percentage relative cell viability was then calculated from Eq. (2):

$$\text{Percentage relative cell viability} = (A \times 100)/B \quad (2),$$

where A and B are the A_{540} with and without the test compound.

Cellular TYR activity assay

Cellular TYR activity was assayed as the DOPA oxidase activity. The B16F10 melanoma cells were divided into groups as mentioned in the 'cell viability assay' section, except that they were cultured in a 25-cm^2 cell culture flask at a density of 5 x 10^5 cells/5 mL CM, and incubated as mentioned in the 'cell viability assay' section for 24 h, and then treated with various concentrations of the respective test compound for 48 h. The CM was then removed and the cells washed with iced-cold phosphate buffered saline (PBS) and lysed with 500 µL of 0.01 M phosphate buffer (pH 7.4) containing 1% (v/v) Triton X-100 (Merck, Germany). The mixture was freeze-thawed by incubating at -80 °C for 15 min and then kept at room temperature (RT) for 10 min. The sample was centrifuged at 12,000 x g, RT for 15 min and the supernatant was harvested and kept. Then 10 µL of freshly prepared substrate solution (15 mM L-DOPA in 50 mM sodium phosphate buffer, pH 7.4) was added to 90 µL of the supernatant and incubated at 37 °C for 1 h. The absorbance was then measured at 492 nm (A_{492}) using a microplate reader. The percentage TYR-I activity was calculated using Eq. (1).

Melanin content

The B16F10 melanoma cells were divided into groups as mentioned in the 'cell viability assay' section and cultured and treated with test compounds as mentioned in the 'cellular TYR activity assay' section. After the 48-h treatment, 200 µL of the CM was transferred to a well of 96-well plate, and the amount of melanin in the cell-free CM was spectrophotometrically measured at 405 nm (A_{405}) and was calculated using Eq. (3);

$$\% \text{ Melanin content} = (A \times 100)/B \quad (3),$$

where A and B are the A_{405} of the sample and control, respectively.

Transcript expression level of genes involved in melanogenesis

The transcript expression level of the *TYR*, *TRP-1*, and *TRP-2* genes was evaluated by one-stage quantitative real-time reverse transcriptase (qrtRT)-PCR. B16F10 melanoma cells (5 x 10^5 cells/5 mL) were cultured as mentioned in the 'cellular TYR activity assay' section. After 48 h exposure to the respective compound (22 µM horsfieldone A, 14 µM maingayone D, or 1.407 mM kojic acid), cultured cells were collected by trypsinization, washed with PBS and harvested by centrifugation at 15,000 x g, RT for 5 min. After cell harvesting, total RNA from each sample was extracted using an RNeasy® Mini Kit (Catalog No. 74104; Qiagen, USA). The concentration of the eluted RNA was spectrometrically measured at 260 nm. In addition, the purity of RNA was determined from the ratio of absorbance at 260 and 280 nm. Next, the extracted RNA was stored at -20 °C until used.

The qrtRT-PCR was performed using the One-Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time; Catalog No. R086A; Takara, Japan). The qrtRT-PCR mixture contained 5 µL of total RNA (20 ng RNA), 0.4 µL each of the forward and reverse primers, 0.5 µL PrimeScript™ Enzyme Mix, and 2x One-Step SYBR® RT-PCR Buffer. The nucleotide sequences of the primers used in this work are from Kim *et al*. [22], while the transcript level of the *glyceraldehyde-3-phosephate dehydrogenase* (*GAPDH*) gene was used as the internal control. All nucleotide sequences of the used primers are listed in Table 1.

The amplification and quantification of each gene of interest was performed using the CFX96™ real-time PCR detection system (Bio-Rad, USA). Thermocycling was performed at 50 °C for 2 min, 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 50 °C for 1 min [22]. The expression level of all target genes was normalized to that of *GAPDH* in each sample and compared as the relative expression level between the control and treated samples.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD), derived from three independent repeats in each experiment. The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test for the significance of differences between the means. Significance was accepted at the $p < 0.05$ level. All analyses were performed using the SPSS version 22 program.

Table 1. Targeted genes and primers used for their amplification by qrtRT-PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
GADPH	GGGCATCCTGGGCTACTCTG	GAGGTCCACCACCCTGTTGC
TYR	GGGCCCAAATTGTACAGAGA	ATGGGTGTT GACCATTGTT
TRP-1	GTTCAATGGCCAGGTCAGGA	CAGACAAGAAGCAACCCCGA
TRP-2	GCTTGGAGCAGCAAGACAAG	ATTACACAGTGTGACCCGGC

Table 2. The percentage TYR-I activity of horsfieldone A, maingayone D, and kojic acid.

Compound	Percentage TYR-I activity		
	0.1 mM	0.2 mM	0.4 mM
Horsfieldone A*	16.32 ± 0.74^{a}	35.49 ± 1.11^{b}	NI
Maingayone D	85.64 ± 0.97	NI	NI
Kojic acid	71.84 ± 5.38^{a}	83.80 ± 0.61^{b}	89.7 ± 1.11^{b}

Remark: NI represents no detected inhibition. *0.3 mM horsfieldone A has a TYR-I activity of 50.34 ± 1.50%. Data are shown as the mean ± SD, derived from three experiments. Means with a different letter are significantly different ($p < 0.05$; Post-hoc test).

RESULTS

Primary screening of selected compounds

Horsfieldone A, maingayone D, and kojic acid were tested for TYR-I activity using the *in vitro* mushroom TYR-I assay. The results are summarized in Table 2 and Fig. 1.

At a concentration of 0.1 mM, maingayone D showed the highest TYR-I activity against mushroom TYR at 85.64 ± 0.97%, compared to kojic acid at 71.84 ± 5.38%. It was not possible to measure the A_{475} of the reactions with 0.2 and 0.4 mM maingayone D or 0.4 mM horsfieldone A because of precipitation in the reaction. However, at these concentrations, kojic acid was the most potent TYR-I, inhibiting the mushroom TYR activity in a dose-dependent manner.

From the TYR-I activity *vs*. concentration (Table 2 and Fig. 1), it was clear that horsfieldone A and maingayone D exhibited a TYR-I activity of more than 30%, but at concentrations up to 0.1 M horsfieldone A attained less than 17% TYR-I activity compared to more than 70% and 85% for kojic acid and maingayone D, respectively. However, horsfieldone A had a TYR-I activity of 50.34 ± 1.50% at 0.3 mM.

From the data in Table 2 and Fig. 1, the IC_{50} values were estimated with maingayone D having the lowest IC_{50} value (20 ± 1 μM), followed by kojic acid (48 ± 6μM) and horsfieldone A (294 ± 9 μM). All three IC_{50} values were significantly different ($p < 0.05$; One-way ANOVA).

Effect of selected compounds on cell viability

The relative cell viability was investigated using the MTT assay, where MTT (yellow tetrazole) is reduced by NAD(P)-dependent cellular oxidoreductase enzymes in viable (mitochondrial metabolically active) cells to insoluble formazan (purple color). The amount of purple product is, therefore, broadly proportional to the number of viable cells, and so in a short term assay (less than the cell cycle time) it can be used to measure the relative viability. In this study it was used to measure the cytotoxic effect of the selected compounds by investigating the reduced relative cell viability of B16F10 melanoma cells.

The effect of a 72-h exposure to horsfieldone A on the morphology of B16F10 melanoma cells is shown in Fig. 2. The B16F10 melanoma cells in the control group were mixed between spindle-shaped and epidermal-like cells, in close contact

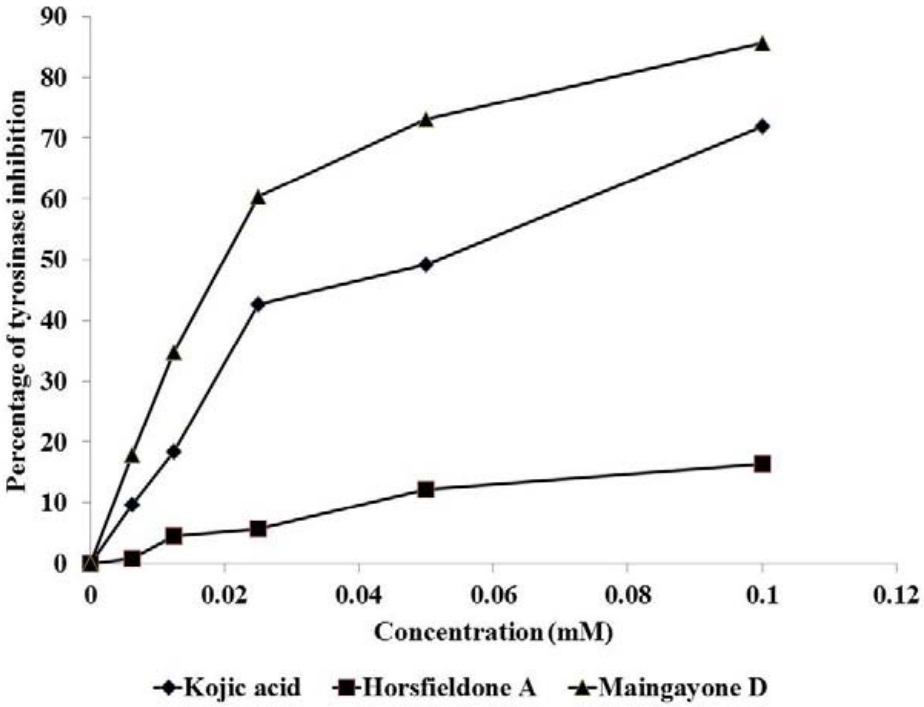

Fig. 1. The TYR-I activity of horsfieldone A, maingayone D, and kojic acid. Data are shown as the mean ± SD, derived from three experiments. Means with a different letter are significantly different ($p < 0.05$; Post-hoc test).

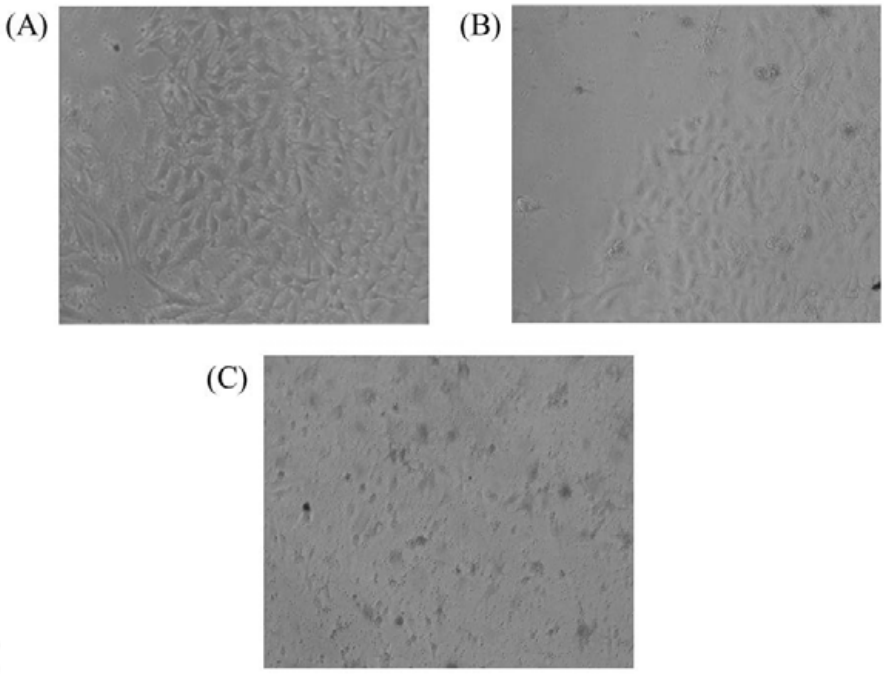

Fig. 2. Effect of horsfieldone A on the morphology of B16F10 melanoma cells under light microscopy (200X): (A) control, (B) B16F10 melanoma cells with 11 μM horsfieldone A, (C) B16F10 melanoma cells with 22 μM horsfieldone A. Images are representative of those seen from 5 fields of view per experiment and three independent experiments.

with neighboring cells, and strongly adhered to the surface of the well plate. Morphological changes were clearly detected after exposure of horsfieldone A at 22 µM, where a loss of contact between cells, cell shrinkage with rounded or irregular cell outlines, more dendrites, and a marked decrease in the number (density) of adherent cells were evident. Obviously, horsfieldone A did not have a significant cytotoxic effect until at 11 µM (cell viability of 87.97 ± 3.88%, Fig. 3B), while at 22 µM and above it showed cytotoxic effect with a relative cell viability of 42.37 ± 17.41% at 22 µM that decreased at higher concentrations in a dose-dependent manner (Fig. 3A). Thus, horsfieldone A at 0-11 µM was selected for investigating the effect on melanogenesis.

Likewise, the cytotoxic effect of maingayone D at 4 and 7 µM on B16F10 melanoma cells was evaluated by cell morphology and relative cell viability. After a 72-h treatment, the B16F10 melanoma cells were observed under light microscopy at 200X magnification (Fig. 4). B16F10 melanoma cells in the control group and those treated with 4 µM maingayone D were similar, showing a mixture of spindle-shaped and epidermal-like cells, in close contact with neighboring cells, and adhered to the surface of the well plate. However, at 7 µM maingayone D induced clear morphological changes in the B16F10 cells (Fig. 4C), with a slight loss of contact between cells, cell shrinkage, an irregular cell outline, and more dendrites. The relative cell viability was significantly decreased by maingayone D in a concentration-dependent manner at concentrations above 4 µM, but with no significant cytotoxic effect at lower concentrations (relative cell viability at 4 µM of 80.63 ± 2.33%). Thus, maingayone D at a concentration range from 0-4 µM was chosen for further experiments.

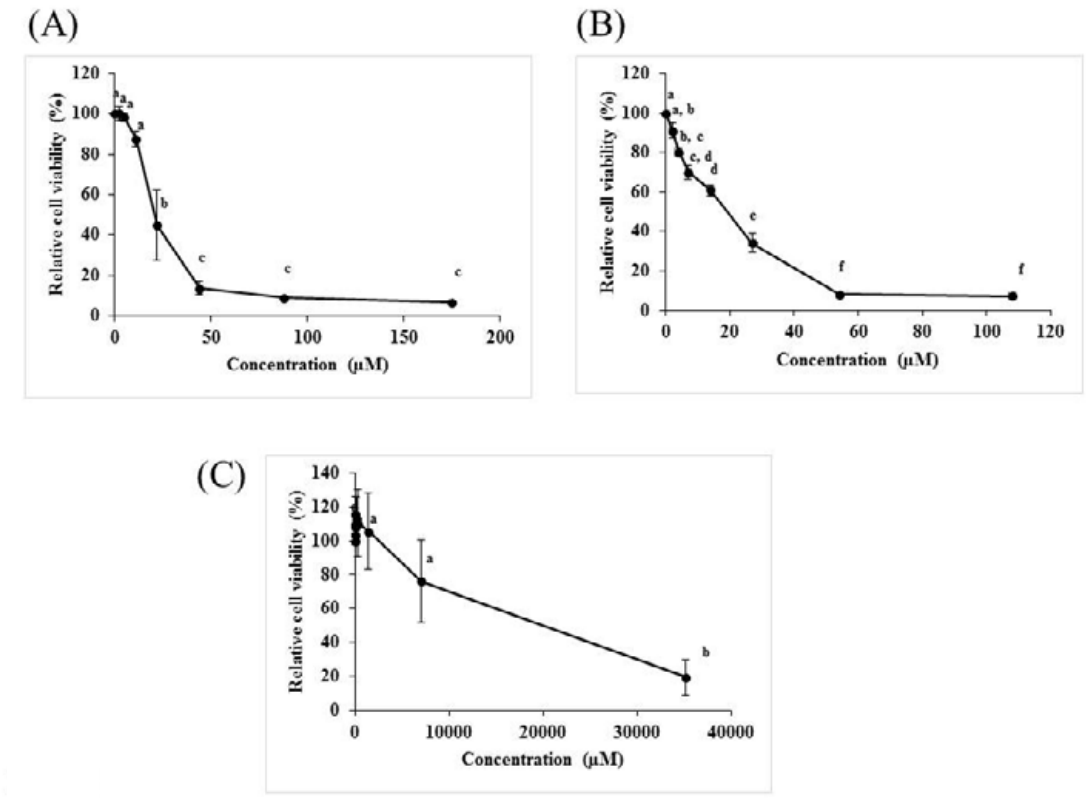

Fig. 3. Effect of a 72-h exposure to (A) horsfieldone A, (B) maingayone D, and (C) kojic acid at various concentrations on the relative cell viability of B16F10 melanoma cells (control group = 100%), as determined using the MTT assay. Data are shown as the mean ± SD from three independent experiments. Means with a different superscript letter are significantly different ($p < 0.05$; Post-hoc test).

For kojic acid, after the 72-h treatment the B16F10 melanoma cells were observed under light microscopy at 200X magnification (Fig. 5). As before, the B16F10 melanoma cells in the control group were mixed between spindle-shaped and epidermal-like cells, in close contact with neighboring cells, and adhered to the surface of the well plate. Morphological changes were clearly detected after exposure to kojic acid at 1,407 µM, with a slight loss of contact between cells, cell shrinkage, and irregular cell outlines. At a higher concentration of 7037 µM the B16F10 melanoma cells also showed a slight cell shrinkage, irregular cell outline, and a greater loss of contact between cells. No significant reduction in the relative cell viability (cytotoxic effect) was observed up to 1,407 µM (cell viability of 100%), but at 7.04 and 35.18 mM a clear cytotoxic effect of kojic acid was noted with the relative cell viability being reduced to 76.10 ± 24.58 and 19.35 ± 10.43%, respectively. Thus, kojic acid at concentrations ranging from 0-1,407 µM was selected for the next experiments.

The cytotoxicity IC_{50} values for each compound against B16F10 melanoma cells are summarized in Table 3, which shows that horsfieldone A and maingayone D were much more cytotoxic (lower IC_{50} value) than kojic acid.

The cellular TYR-I activity of the selected compounds

The *in vitro* inhibitory effect of the selected compounds on the B16F10 cellular TYR activity was investigated *via* measuring the rate of L-DOPA oxidation. B16F10 melanoma cells were incubated with the respective target compound at its highest non-cytotoxic concentration (80% relative cell viability) for 48 h, and then the percentage inhibition of TYR activity (i.e., TYR-I activity) was calculated and recorded.

For horsfieldone A, all chosen concentrations of horsfieldone A did not significantly suppress the cellular TYR activity (Fig. 6A), with a numerical but not significant TRY-I activity (2.58%) at 22 µM, even though at this concentration of horsfieldone A

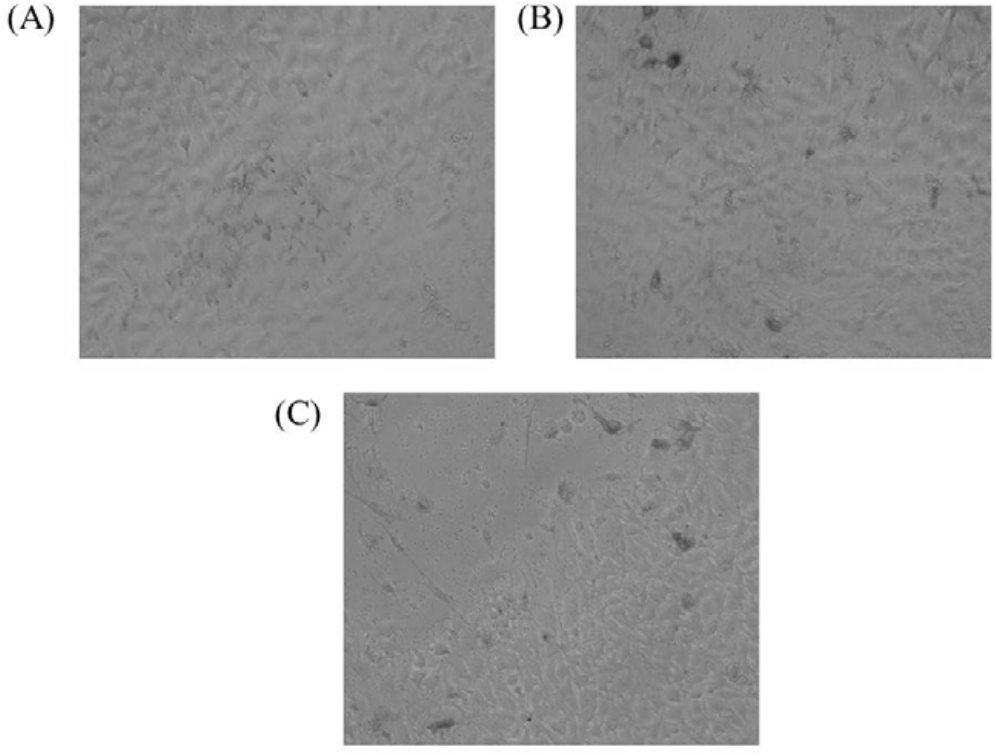

Fig. 4. Effect of maingayone D on the morphology of B16F10 melanoma cells under light microscopy (200X) after 72-h treatment with: (A) 0 µM (control), (B) 4 µM, and (C) 7 µM maingayone D. Images are representative of at least 5 fields of view per sample and from three independent experiments.

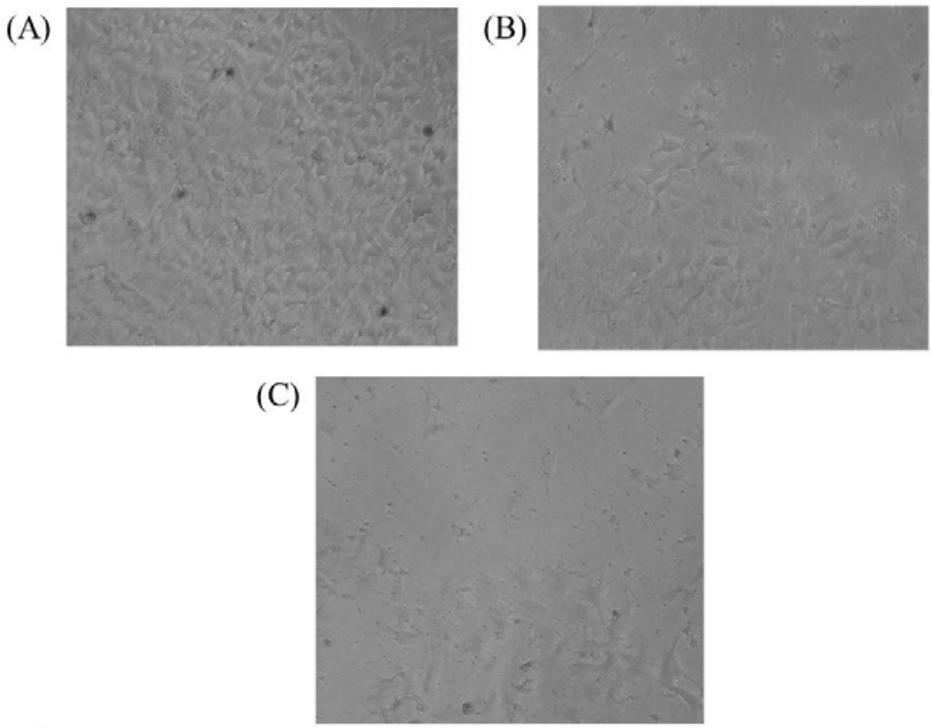

Fig. 5. Effect of a 72-h kojic acid treatment at (A) 0 µM (control), (B) 1.407 mM, and (C) 7.04 mM on the morphology of B16F10 melanoma cells under light microscopy (200X). Images are from at least 5 fields of view per treatment and three independent experiments.

Table 3. The cytotoxic IC_{50} values of horsfieldone A, maingayone, D and kojic acid against B16F10 cells.

Compound	IC_{50} (µM)
Horsfieldone A	20.51 ± 0.001^{a}
Maingayone D	19.20 ± 0.004^{a}
Kojic acid	$10{,}764.90 \pm 0.555^{b}$

Data are shown as the mean ± SD, derived from three experiments. Means with a different superscript letter are significantly different ($p < 0.05$; One-way ANOVA).

the relative cell viability was decreased to only 44.79%.

For maingayone D at 1, 2, and 4 µM, the cellular TYR activity is shown in Fig. 6B, where at each concentration maingayone D did not show any significant TYR-I activity. Moreover, maingayone D also showed no TYR-I activity even at 14 µM, which was cytotoxic and reduced the relative survival rate of the cells to 60.86%.

For kojic acid, screened at 11.2, 56, 281, and 1,407 µM for 48 h, the cellular TYR activity relative to the control (set at 100%) is shown in Fig. 6C. Kojic acid displayed a dose-dependent TYR-I activity in B16F10 melanoma cells, and this was significant at 1,407 µM, but at 11.2, 56, and 281 µM there was no TYR-I activity. Thus, kojic acid at 1,407 µM was used for estimating the effect of this compound on melanogenic gene expression.

Effect of the selected compounds on the melanin content

To determine the ability of the selected compounds to inhibit melanogenesis, melanin synthesis in B16F10 melanoma cells was examined. Cells were incubated with each compound (or just the DMSO solvent for the control) for 48 h and the melanin contents were compared relatively to that for the control (set as 100% melanin content). Kojic acid served as the positive control for inhibiting melanin content.

After 48 h of incubation with horsfieldone A, secreted melanin in the CM was clearly observed and was not significantly different from that of the control group (Fig. 7A), except for the group treated

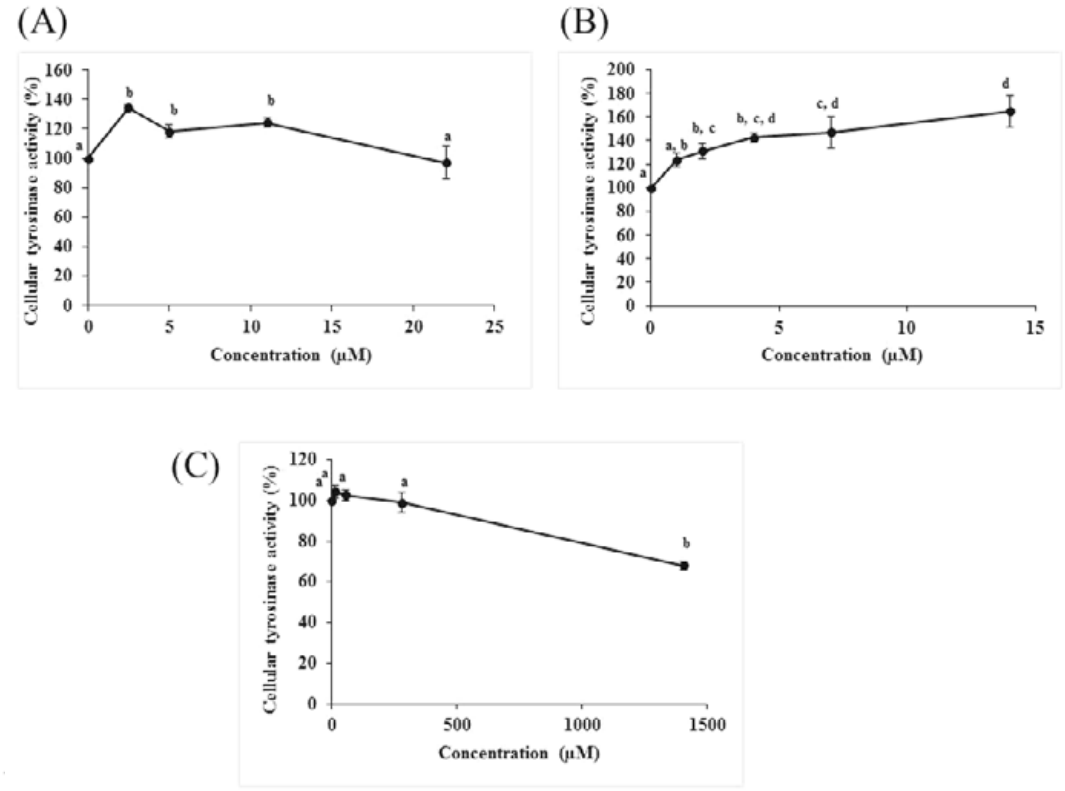

Fig. 6. Effect of (A) horsfieldone A, (B) maingayone D, and (C) kojic acid treatment for 48 h on the cellular TYR activity of B16F10 melanoma cells. The TYR activity of treated cells is reported to that of the control group (set at 100%). Data are shown as the mean ± S.D. from three independent experiments. Means with a different superscript letter are significantly different between groups ($p < 0.05$; Post-hoc test).

with 22 μM horsfieldone A that had a slightly lighter color for the CM (less melanin) than the control group. Thus, horsfieldone A at 0, 2.5, 5, and 11 μM showed no inhibitory activity on melanin production, whereas at 22 μM weak but significant inhibitory activity of 11.15 ± 0.48% was noted. However, horsfieldone A at 22 μM was cytotoxic, reducing the relative cell viability of B16F10 melanoma cells to 44.79%. Regardless, as the lowest concentration to induce inhibition of melanin synthesis, 22 μM horsfieldone A was then used to evaluate the effect of horsfieldone A on gene expression.

For maingayone D, after 48 h of exposure, secreted melanin could still be observed and the color of the CM in the treated groups was not significantly different from the control group except for at 14 μM maingayone D, where the color of the CM was lighter than that of the control group (Fig. 7B). Overall, maingayone D showed no inhibitory activity on melanin production at concentrations less than 7 μM, whereas 14 μM of maingayone D showed a significant inhibitory activity at 29.30 ± 1.21%. Although maingayone D at 14 μM was cytotoxic (relative cell viability of B16F10 cells of 60.86%), this was the lowest concentration that induced melanin synthesis inhibition and so was used for evaluating the effect of maingayone D on gene expression.

For kojic acid, after 48 h of incubation secreted melanin could be observed in the CM but at a lower level (lighter colour) than that of the control group. The amount of melanin was significantly decreased in a dose-dependent manner (Fig. 7C) ranging from 3.03 ± 1.09% inhibition at 11.2 μM up to 31.09 ± 1.24% at 1,407 μM kojic acid without significant cytotoxicity and so 1,407 μM kojic acid was used to evaluate its effect on gene expression.

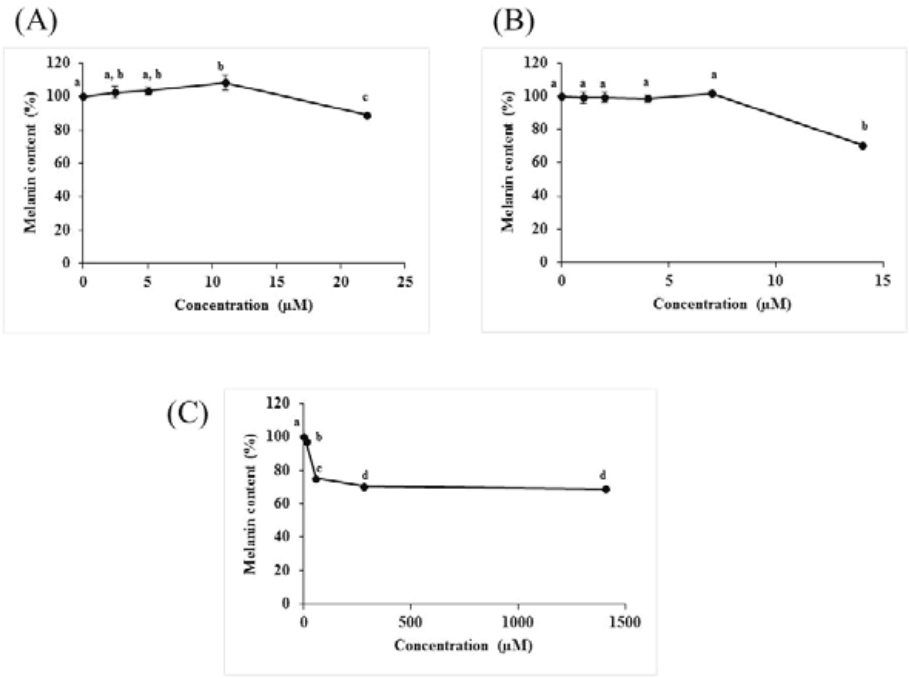

Fig. 7. Effect of a 48-h exposure to various concentrations of (A) horsfieldone A, (B) maingayone D, and (C) kojic acid on the melanin content of cultured B16F10 melanoma cells. The melanin content is reported as relative to that of the control group (set at 100%). Data are shown as the mean ± S.D. from three independent experiments. Means with a different superscript letter are significantly different ($p < 0.05$; Post-hoc test).

Effect of the selected compounds on the transcriptional expression of genes involved in melanogenesis

There are three known important enzymes in melanogenesis: TYR, TRP-1, and TRP-2. Of these, TYR is the most important enzyme because it is the rate-limiting enzyme in melanogenesis and catalyzes the hydroxylation of tyrosine into L-DOPA, which is later oxidized to DOPAquinone. The enzymes TRP-1 and TRP-2 are required for eumelanin synthesis, where TRP-1 catalyzes the oxidation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to indole-5,6-quinone carboxylic acid in the presence of bound Cu^{2+} ions, but not in the presence of Zn^{2+}, while TRP-2 tautomerizes DOPAchrome into DHICA.

The effect of a 48-h treatment of B16F10 cells with horsfieldone A (22 μM), maingayone D (14 μM), or kojic acid (1.407 mM) on the transcript expression level of *TYR*, *TRP-1*, and *TRP-2* was investigated by qrtRT-PCR. These respective concentrations were chosen because they showed promising inhibitory activity on mushroom TYR and B16F10 melanin production. After treatment, total RNA was isolated from the cell lysate and subjected to one-stage qrtRT-PCR using gene fragment specific primers, with *GAPDH* as the internal reference control.

For horsfieldone A, the transcript expression level of *TYR*, *TRP-1*, and *TRP-2* relative to that of *GAPDH* is shown in Fig. 8A. After horsfieldone A treatment, the *TRP-2* transcript expression level was down-regulated, while the *TYR* and *TRP-1* transcript levels were up-regulated. These results suggested that horsfieldone A might reduce melanin production in B16F10 melanoma cells by down-regulating *TRP-2* at the transcriptional level.

For maingayone D at 14 μM, the relative transcript expression level of *TYR* and *TRP-2* was down-regulated, while *TRP-1* was up-regulated (Fig. 8B). Thus, maingayone D likely inhibited *TYR* and *TRP-2* at the transcription level in B16F10 melanoma cells.

For kojic acid at 1,407 μM, the relative *TRP-1* transcript level was down-regulated, while *TYR* and *TRP-2* were up-regulated (Fig. 8C). Thus, kojic acid may reduce melanin synthesis in B16F10

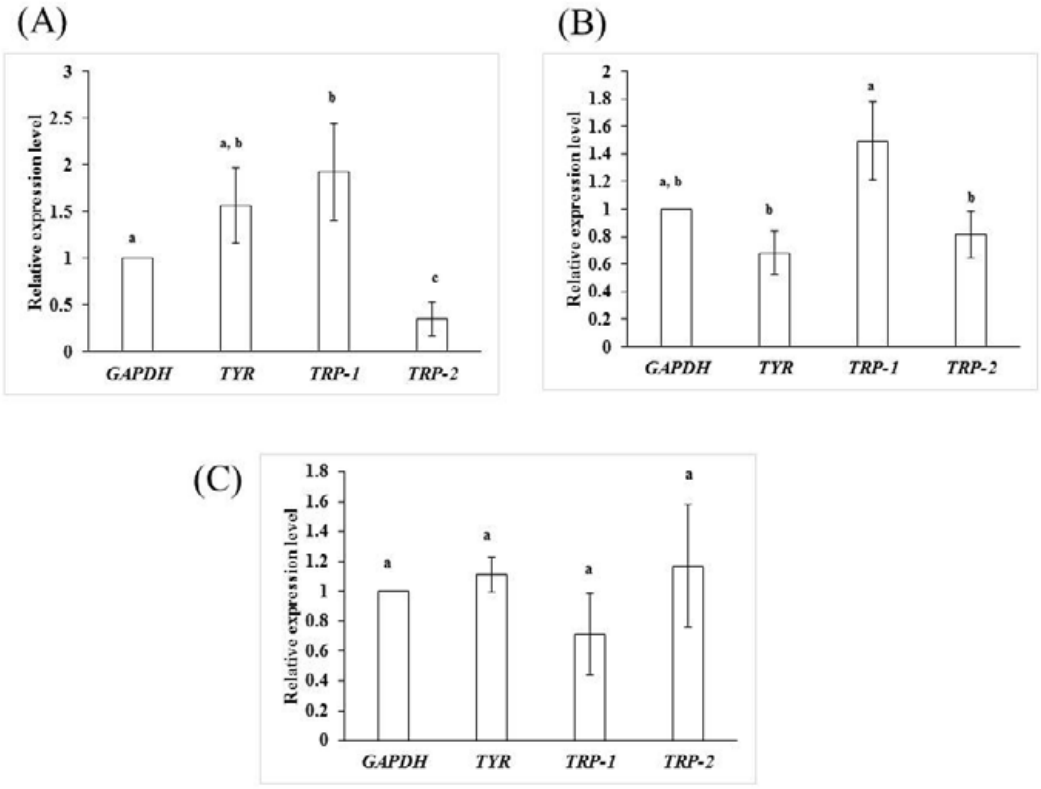

Fig. 8. Effect of (A) 22 μM horsfieldone A, (B) 14 μM maingayone D, and (C) 1,407 μM kojic acid treatment for 48 h on the transcript level of three genes involved in melanogenesis (TYR, TRP-1, and TRP-2). Transcript levels were examined by qrtRT-PCR and are shown relative to that for GAPDH as an internal control. Data are shown as the mean ± SD from three independent experiments. Means with a different superscript letter indicate a significant difference between groups ($p < 0.05$; Post-hoc test).

melanoma cells by down-regulating *TRP-1* gene expression.

DISCUSSION

Melanin is responsible for protecting the skin from the damage due to ultraviolet rays and eliminating ROS, but excessive accumulation of melanin causes hyperpigmentation and various skin disorders, such as melasma, freckles, post-inflammatory melanoderma, and solar lentigo. Therefore, application of TYR-Is is the most common way to reduce pigmentation in the skin. Thus, searching for new agents from natural products with a safer but more effective potential without side effects is still required.

In this research, mushroom TYR was used because it is highly homologous with mammalian TYRs [10]. Here, horsfieldone A and maingayone D, new arylalkanones isolated from *Horsfieldia motley* [23], showed TYR-I activity (Fig. 1). Considering the relationship between the chemical structure and TYR-I activity, maingayone D has the same arylalkanones' moieties as horsfieldone A (Fig. 9). However, the presence of an additional phenolic moiety in maingayone D is an important difference from horsfieldone A. As a consequence, maingayone D also has two more hydroxyl groups than horsfieldone A. This data is consistent with Saghaie *et al*. [24], who reported that some novel derivatives of kojic acid containing two free hydroxyl groups were more potent than their analogues with one hydroxyl group.

Furthermore, other studies have demonstrated that TYR-I activity might depend on the number of hydroxyl groups in the phenolic compounds that can form a hydrogen bond to the active site of TYR. These TYR-Is bind to the active site of TYR,

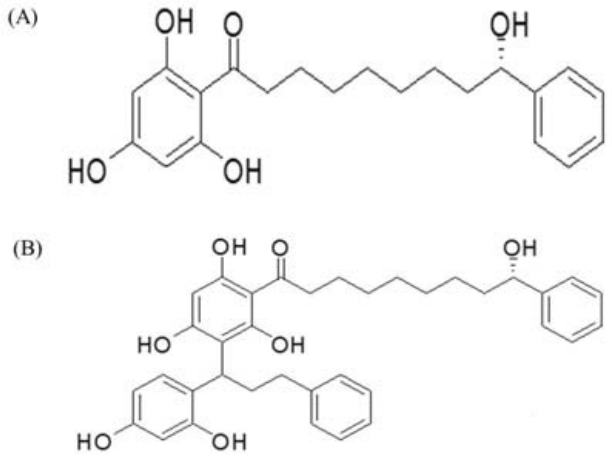

Fig. 9. Chemical structure of (A) horsfieldone A and (B) maingayone D.

which consists of two copper ions surrounded by three histidine residues, leading to a lower enzymatic activity, steric hindrance, or changed conformation [25-27]. Moreover, the location of the hydroxyl groups is also an important factor in their TYR-I efficacy [28, 29]. Thus, the hydroxyl moiety and their location determine their effectiveness as a functional group for the inhibition of TYR.

For cell viability and cellular TYR activity, B16F10 mouse melanoma cells were used as a model because this cell line produces melanin, possesses TYR activity and is easy to culture *in vitro* [30]. Although horsfieldone A and maingayone D were cytotoxic (Fig. 3), at low concentrations, horsfieldone A could suppress the cellular TYR activity, unlike kojic acid and maingayone D (Fig. 6). Although TYR from different sources has conserved sequences, they still have different amino acids and molecular weights [31]. Maeda and Fukuda [32] suggested that the inhibition of mushroom TYR activity does not correlate with cellular TYR activity or melanin production in cultured melanocytes. In addition, Mann *et al.* [33] reported that thiamidol was the most potent inhibitor of human TYR (IC_{50} = 1.1 μM) but inhibited mushroom TYR only weakly (IC_{50} = 108 μM), which reflected that mushroom TYR had different amino acid sequences in the region of the active site than human TYR. Furthermore, *p*-coumaric acid was found to be a stronger inhibitor of human or murine than mushroom TYRs [34]. Therefore, it is highly possible that horsfieldone A and maingayone D have a TYR-I activity against mushroom TYR but a different ability to inhibit TYR from B16F10 mouse melanoma cells. However, both showed a slight inhibitory effect on the melanin content in B16F10 mouse melanoma cells (Fig. 7).

Melanin is the end product of melanogenesis, which requires TYR, TRP-1, and TRP-2 expression. The transcript expression level of these three genes, relative to that of *GAPDH*, was evaluated by qrtRT-PCR. The level of *TRP-2* transcripts was decreased by horsfieldone A, while *TYR* and *TRP-1* were up-regulated (Fig. 8). These results suggested that horsfieldone A reduced melanin content in B16F10 melanoma cells by down-regulating *TRP-2* at the transcriptional level. In contrast, maingayone D decreased melanin synthesis by down- regulating *TYR* and *TRP-2* transcript levels, but did not significantly change the transcript levels of *TRP-1*. Several reports have demonstrated that some compounds have an anti-melanogenesis activity through inhibition of *TYR* expression, even though they had no detectable direct TYR-I activity in general [35]. For example, acetylsalicylic acid exerted a strong dose-dependent inhibitory effect on *TYR* expression in B16 melanoma cells, while it did not exert any effect on mushroom TYR activity [36]. Osthol was found to decrease *TYR*, *TRP-1*, and *TRP-2* expression, but not to exhibit TYR-I activity [37]. Soyasaponin Ag inhibited *TRP-2* expression, although it had no intracellular TYR-I activity [38].

Additionally, it is known that the *TYR*, *TRP-1*, and *TRP-2* genes are coordinately regulated by the micropthalmia (MITF) transcription factor, which binds to the M-box sequences of these genes with different affinities. Moreover, several lines of evidence indicate that these genes are also regulated independently of each other as well as independently of MITF [39]. Therefore, it is possible that maingayone D can inhibit melanin production by controlling *TYR* and *TRP-2* expression.

CONCLUSION

In conclusion, horsfieldone A and maingayone D are potential *in vitro* TYR-Is. However, compared to kojic acid, horsfieldone A and maingayone D are cytotoxic to B16F10 melanoma cells. Thus, the effective application concentration/duration of both compounds is of concern for potential use. Horsfieldone A and maingayone D could reduce the melanin content but not the cellular TYR activity. Moreover, horsfieldone A reduced the mRNA expression of *TRP-2*, while maingayone D significantly reduced the mRNA expression of *TYR* and *TRP-2*. The results implied that horsfieldone A and maingayone D are potential melanin synthesis inhibitors.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing financial interests.

REFERENCES

1. Boo, Y. C. 2019, Antioxidants, 8, 332.
2. Stulberg, D. L., Clark, N. and Tovey, D. 2003, Am. Fam. Physician., 68, 1955-1960.
3. Nieuweboer-Krobotova, L. 2013, J. Eur. Acad. Dermatol. Venereol., 27, 2-4.
4. Vashi, N. A. and Kundu, R. V. 2013, Br. J. Dermatol., 169, 41-56.
5. D' Mello, S. A., Finlay, G. J., Baguley, B. C. and Askarian-Amiri, M. E. 2016, Int. J. Mol. Sci., 17, 1144.
6. Cichorek, M., Wachulska, M., Stasiewicz, A. and Tyminska, A. 2013, Postepy Dermatol. Alergol., 30, 30-41.
7. Pillaiyar, T., Manickam, M. and Namasivayam, V. 2017, J. Enzyme Inhib. Med. Chem., 32, 403-425.
8. Gurme, S. T., Surwase, S. N., Aware, C. B., Vyavahare, G. D. and Jadhav, J. P. 2020, JBAPN., 10, 233-249.
9. Halaouli, S., Asther, M., Sigoillot, J. C., Hamdi, M. and Lomascolo, A. 2006, J. Appl. Microbiol., 100, 219-232.
10. Chang, T. S. 2009, Int. J. Mol. Sci., 10, 2440-2475.
11. Panzella, L. and Napolitano, A. 2019, Cosmetics., 6, 57.
12. Zolghadri, S., Bahrami, A., Hassan Khan, M.T., Munoz-Munoz, J., Garcia-Molina, F., Garcia-Canovas, F. and Saboury, A. A. 2019, J. Enzyme Inhib. Med. Chem., 34, 279-309.
13. Mishima, Y., Ohyama, Y., Shibata, T., Hatae, S. and Seto, H. 1994, Skin Res., 36, 134-150.
14. Desmedt, B., Courselle, P., De Beer, J. O., Rogiers, V., Grosber, M., Deconinck, E. and De Paepe, K. 2016, J. Eur. Acad. Dermatol. Venereol., 30, 943-950.
15. Saeedi, M., Eslamifar, M. and Khezri, K. 2019, Biomed. Pharmacother., 110, 582-593.
16. Crespo, M. I., Chaban, M. F., Lanza, P. A., Joray, M. B., Palacios, S. M., Vera, D. M. A. and Carpinella, M. C. 2019, Food Chem. Toxicol., 125, 383-391.
17. Lee, G. Y., Cho, B. O., Shin, J. Y., Jang, S. I., Cho, I. S., Kim, H. Y., Park, J. S., Cho, C. W., Kang, J. S., Kim, J. H. and Kim, Y. H. 2018, Arch. Pharm. Res., 41, 490-496.
18. Quispe, Y. N., Hwang, S. H., Wang, Z. and Lim, S. S. 2017, Molecules., 22, 402.
19. Ko, Y. J., Yang, S. K., Song, S. M., Yoon, W. J. and Bae, K. H. 2015, JBAPN., 5, 12-17.
20. Ramadhan, R. and Phuwapraisirisan, P. 2015, Nat. Prod. Commun., 10, 325-328.
21. Momtaz, S., Lall, N. and Basson, A. 2008, S. Afr. J. Bot., 74, 577-582.

22. Kim, K., Leutou, A. S., Jeong, H., Kim, D., Seong, C. N., Nam, S. J. and Lim, K. M. 2017, Mar. Drugs., 15, 138.
23. Ramadhan, R. and Phuwapraisirisan, P. 2015, Bioorganic Med. Chem. Lett., 25, 4529-4533.
24. Saghaie, L., Pourfarzam, M., Fassihi, A. and Sartippour, B. 2013, Res. Pharm. Sci., 8, 233-242.
25. Masum, M. N., Yamauchi, K. and Mitsunaga, T. 2019, Rev. Agric. Sci., 7, 41-58.
26. Qian, W., Liu, W., Zhu, D., Cao, Y., Tang, A., Gong, G. and Su, H. 2020, Exp. Ther. Med., 20, 173-185.
27. Yoon, K. N., Alam, N., Lee, K. R., Shin, P. G., Cheong, J. C., Yoo, Y. B. and Lee, T. S. 2011, Molecules., 16, 2334-2347.
28. Promden, W., Viriyabancha, W., Monthakantirat, O., Umehara, K., Noguchi, H. and De-Eknamkul, W. 2018, Molecules., 23, 1403.
29. Zuo, A. R., Dong, H. H., Yu, Y. Y., Shu, Q.L., Zheng, L. X., Yu, X. Y. and Cao, S. W. 2018, Chin. Med., 13, 51.
30. Chatatikun, M., Yamauchi, T., Yamasaki, K., Aiba, S. and Chiabchalard, A. 2019, J. Tradit. Complement. Med., 9, 66-72.
31. Seo, S. Y., Sharma, V. K. and Sharma, N. 2003, J. Agric. Food Chem., 51, 2837-2853.
32. Maeda, K. and Fukuda, M. 1996, J. Pharmacol. Exp. Ther., 276, 765-769.
33. Mann, T., Gerwat, W., Batzer, J., Eggers, K., Scherner, C., Wenck, H., Stab, F., Hearing, V. J., Rohm, K. H. and Kolbe, L. 2018, J. Invest. Dermatol., 138, 1601-1608.
34. An, S., Koh, J. S. and Boo, Y. C. 2010, Phytother. Res., 24, 1175-1180.
35. Chung, S. Y., Seo, Y. K., Park, J. M., Seo, M. J., Park, J. K., Kim, J. W. and Park, C. S. 2009, Biosci. Biotechnol. Biochem., 73, 1704-1710.
36. Sato, K., Takahashi, H., Iraha, R. and Toriyama, M. 2008, Biol. Pharm. Bull., 31, 33-37.
37. Kim, S. B., Kim, C. T., Liu, Q., Jo, Y. H., Choi, H. J., Hwang, B. Y., Kim, S. K. and Lee, M .K. 2016, Pharm. Biol., 54, 1373-1379.
38. Yang, S. H., Tsatsakis, A. M., Tzanakakis, G., Kim, H. S., Le, B., Sifaki, M., Spandidos, D. A., Tsukamoto, C., Golokhvast, K. S., Izotov, B. N. and Chung, G. 2017, Inter. J. Mol. Med., 40, 631-636.
39. Moleephan, W., Wittayalertpanya, S., Ruangrungsi, N. and Limpanasithikul, W. 2012, Asian Biomed., 6, 413-422.