Effect of Plant Compounds on *in vitro* Tyrosinase Activity and Cell Viability of B16F10 Melanoma Cells

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Abstract

Tyrosinase inhibitor plays an important role in anti-melanogenesis, resulting in lighter looking skin. Thus, this work was aimed to test the efficacy of compounds from some plants which affect *in vitro* tyrosinase inhibition and cell viability of B16F10 melanoma cells after 72 h treatment. Fifteen compounds from 6 different plants including pinostrobin, pinocembrin, cardamomin and alpinetin from *Boesenbergia rotunda*, piperine from *Piper nigrum*, rosmarinic acid from *Orthosiphon aristatus*, methyl lansioside C, lansioside C and lansioside B from *Lansium parasiticum*, sesamin, sesamolin and samin from *Sesamum indicum*, malabaricone A, horsfieldone A and maingayone D from *Horsfieldia motley* were used. To determine the effect of plant compounds on melanogenesis, tyrosinase activity was evaluated *in vitro*. The three most effective compounds were used to test for cell cytotoxicity on B16F10 melanoma cells. The results revealed that piperine, horsfieldone A and maingayone D had high tyrosinase inhibition with IC_{50} values of 526, 294 and 38 µM, respectively. In cytotoxicity test, IC_{50} values of piperine, horsfieldone A, maingayone D and kojic acid were 79, 21, 19 and 10,765 µM, respectively. From the mentioned data, it suggests that piperine, horsfieldone A and maingayone D possess potential for tyrosinase inhibitors.

Keywords**:** *Cell viability, Melanogenesis, Plant compound, Tyrosinase, Tyrosinase inhibitor*

1. Introduction

Nowadays, skin-caring cosmetics are in the center of attention in various societies in many countries around the world. Especially in Asia, there is a boom in this industry (Chen et al., 2016). Skin whitening products are commercially available for cosmetic purposes in order to obtain a lighter skin appearance. They are also utilized for clinical treatment of pigmentary disorders such as melasma or postinflammatory hyperpigmentation. Whitening agents act as competitive inhibitors of tyrosinase, the key enzyme in melanogenesis (Smith, Vicanova & Pavel, 2009).

Melanogenesis is a biosynthetic pathway for melanin synthesis by melanocytes located in the lowest layer of epidermis in human skin. Melanogenesis is a complex pathway. Three enzymes, tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1) and TRP-2, are important mediators of melanogenesis; in particular, TYR is exclusively necessary for melanogenesis (Han et al., 2015; Pillaiyar et al., 2017). Tyrosinase (polyphenol oxidase, EC 1.14.18.1), a copper-containing oxidase, is the rate-limiting enzyme in melanin biosynthesis. The enzyme catalyzes the hydroxylation of the monophenol L-tyrosine to the o-diphenol 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to the DOPAquinone. The resulting quinone will serve as a substrate for the synthesis of eumelanin and pheomelanin (Chen et al., 2016; Ebanks, Wickett & Boissy, 2009).

Many skin whitening products contain tyrosinase inhibitors. Whitening agents such as ascorbic acid, arbutin, azelaic acid, flavonoids, kojic acid, resveratrol, vitamin E, are widely used in cosmetic products as active substances. Moreover, plant extracts are one of the most popular alternatives because they are safe and have fewer side effects. There is an increasing interest in finding natural tyrosinase inhibitors from them (Ephrem, Elaissari & Greige-Gergesa, 2017).

Mushroom tyrosinase has been widely used as a substitute for mammalian tyrosinase to screen for tyrosinase inhibitors since it is inexpensive and commercially available in a purified form (Seo, Sharma &

Sharma, 2003). For screening target compounds, the popular whitening agents such as kojic acid, arbutin, were used as positive control. As a result, in recent years, many plant extracts of *Morus alba* L. (Moraceae), *Artocarpus heterophyllous*, *Hypericum laricifolium* Juss., etc., have been used as whitening agents (Lee et al., 2002; Momtaz et al., 2008; Nguyen et al., 2016; Quispe et al., 2017).

Boesenbergia rotunda as known as fingerroot is a daily food ingredient and traditional medicinal plant in Southeast Asia and Indo-China. It has been shown to possess anti-allergic, antibacterial, anticancer, anti-inflammatory, antioxidant, antiulcer and antityrosinase activities (Chan et al., 2008; Keonkaew et al., 2011; Ongwisespaiboon & Jiraungkoorskul, 2017). Piper species have high commercial, economic and medicinal importance, particularly for pepper (*Piper nigrum*) in the worldwide spice markets and for its wide range of biological activities (Mukherjee et al., 2001; Ahmad et al., 2012). *Orthosiphon stamineus* is a valuable medicinal plant in traditional folk medicine. Many pharmacological studies have demonstrated the ability of this plant to exhibit antimicrobial, antioxidant, hepatoprotection, antigenotoxic, antiplasmodial, cytotoxic, cardioactive, antidiabetic and anti-inflammatory activities (Ashraf, Sultan & Adam, 2018). *Lansium parasiticum* is a fruit-bearing tree and cultivated widely in Thailand and surrounding countries in the Southeast Asia. The seeds have been traditionally used to treat malaria (Saewan, Sutherland & Chantrapromma, 2006), and the fruit peels are shown α-glucosidase inhibitory activity (Potipiranun, Worawalai, & Phuwapraisirisan, 2018). Furthermore, sesame (*Sesamum indicum*) and *Horsfieldia macrobotrys* are reported to have antioxidant activity (Kumar & Singh, 2014; Ramadhan & Phuwapraisirisan, 2015a, 2015b).

The aim of this work was to determine the tyrosinase inhibitory effect of fifteen compounds from six various plants to develop melanogenesis inhibitors. In addition, the toxic effects of the three most effective compounds on B16F10 melanoma cells were evaluated.

2. Objectives

1. To test the efficacy of compounds from some plants which can affect *in vitro* tyrosinase inhibition
2. To determine the toxic effects of the three most effective compounds on B16F10 melanoma cells

3. Materials and Methods

3.1 Plant compounds preparation

Plant compounds were received from Preecha Phuwapraisirisan and Rico Ramadhan. Fifteen compounds were from 6 various plants including pinostrobin, pinocembrin, cardamomin and alpinetin from *Boesenbergia rotunda*, piperine from *Piper nigrum*, rosmarinic acid from *Orthosiphon aristatus*, methyl lansioside C, lansioside C and lansioside B from *Lansium parasiticum*, sesamin, sesamolin and samin from *Sesamum indicum*, malabaricone A, horsfieldone A and maingayone D from *Horsfieldia motleyi*.

3.2 *In vitro* tyrosinase activity

An *in vitro* mushroom tyrosinase inhibition assay was performed as described previously by Momtaz et al. (2008) with slight modifications. A reaction mixture in each well of a 96-well plate contained 120 µL of 1.5 mM L-DOPA in 80 mM phosphate buffer (pH 6.8) and 40 µL of the same buffer or plant compounds at various doses. After incubation at 25 °C for 10 min, 40 µL of 165 U mushroom tyrosinase in 80 mM phosphate buffer was added. Then, it was further incubated at 25 °C for 5 min. The absorbance was measured at 475 nm by using a microplate reader. The data were expressed as the percentage of inhibition of tyrosinase activity. Kojic acid was used as a standard tyrosinase inhibitor control. The dose inhibition curve was used to calculate the IC_{50} value.

3.3 Cell culture

B16F10 melanoma cell lines were obtained from the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. Cell lines were cultured in Dulbecco's Modified Eagle Medium

(DMEM) and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified 95% air and 5% CO_2 atmosphere as described previously (Han et al., 2015).

3.4 Cell viability assay

B16F10 melanoma cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After 24 h, the cells were treated with various concentrations of plant compounds for 72 h. After the incubation period, the cells were cultured in fresh media. Ten µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay solution (5 mg/mL in normal saline solution) was added to each well and further incubated at 37 °C for 4 h. Next, 150 µL of dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance was measured at 540 nm by using a microplate reader (Han et al., 2015). The survival rate of cells was calculated as followed: % cell survival = (A x 100)/B, where A indicates the absorbance difference between sample and control while B indicates the absorbance without the sample.

3.5 Statistical Analysis

The results were expressed as mean ± standard deviation (SD). The inhibitory concentration (IC_{50}) was calculated by log-Probit analysis.

4. Results and Discussion

4.1 Effect of plant compounds on *in vitro* tyrosinase activity

Tyrosinase is a binuclear copper-containing enzyme that catalyzes two different reactions using molecular oxygen. An initial reaction consists in the hydroxylation of tyrosine to DOPA (monophenolase activity) and diphenolase activity catalyzes the oxidation of DOPA to dopaquinone. For this purpose, L-DOPA was used as a substrate to determine the inhibitory effect of plant compounds on the diphenolase activity of the mushroom tyrosinase. The inhibitory effect of plant compounds on the activity of mushroom tyrosinase for the oxidation of L-DOPA was investigated by UV-Vis spectrophotometer (Jiménez-Pérez et al., 2018). The effect on tyrosinase inhibition of plant compounds was investigated at a concentration of 0.2 mM, and the result was reported in Table 1. Kojic acid was used as positive control.

Table 1 Percentage inhibition of plant compounds at concentration of 200 µM evaluated by mushroom tyrosinase inhibition activity

Name of Compounds	Sources	Inhibition (%)
Pinostrobin		0.93 ± 4.00
Pinocembrin	*Boesenbergia rotunda*	16.78 ± 3.62
Cardamomin		NI*
Alpinetin		3.22 ± 3.41
Piperine	*Piper nigrum*	27.92 ± 1.70
Rosmarinic Acid	*Orthosiphon aristatus*	10.41± 5.00
Methyl Lansioside C		NI*
Lansioside C	*Lansium parasiticum*	NI*
Lansioside B		NI*
Sesamin		9.42 ± 4.92
Sesamolin	*Sesamum indicum*	0.21 ± 3.28
Samin		1.75 ± 3.30
Malabaricone A		NI*
Horsfieldone A	*Horsfieldia motley*	35.57 ± 0.91
Maingayone D	(*macrobotrys*)	NI**
Kojic acid		87.86 ± 2.85

*NI = No inhibition at this concentration

**Tyrosinase was inhibited more than 85% at lower concentration of 100 µM.

Of all the assayed extracts at 200 µM concentration, the tyrosinase inhibition which was higher than 10% was chosen. Five plant compounds exhibit the satisfying anti-tyrosinase activity. They are pinocembrin from *B. rotunda*, piperine from *P. nigrum*, rosmarinic acid from *O. aristatus*, sesamin from *S. indicum* and horsfieldone A from *H. motley*. However, maingayone D shows the highest tyrosinase inhibitory effect on mushroom tyrosinase. It shows the maximum efficacy of 85 % inhibition of tyrosinase

activity at a concentration of 100 µM, although it is not possible to measure the absorbance at a concentration of 200 µM.

As in Table 2, it reveals that piperine, horsfieldone A and maingayone D have high tyrosinase inhibition with IC_{50} values of 526, 294 and 38 µM, respectively. Compared to kojic acid (IC_{50} = 59 µM), maingayone D shows the highest inhibitory effect against mushroom tyrosinase, while piperine and horsfieldone A are less potent.

Table 2 The IC_{50} values of the most effective compounds

Name of Compounds	Sources	IC_{50} value (µM)
Piperine	*Piper nigrum*	526
Horsfieldone A	*Horsfieldia motley*	294
Maingayone D	*Horsfieldia motley*	38
Kojic acid		59

It was previously reported that piperine induced melanogenesis in cultured mammalian melanocytes by inducing morphological alterations in melan-a cells, with more and longer dendrites observed. Also, it induced melanin dispersion in *Rana tigerina* tadpole melanophores (Lin et al., 1999; Sajid & Ali, 2011). On the contrary, it was shown that piperine had tyrosinase inhibition activity. These data suggest the probable of piperine to inhibit tyrosinase activity. For horsfieldone A and maingayone D as new arylalkanones from *Horsfieldia motley*, Ramadhan and Phuwapraisirisan (2015a, 2015b) reported that maingayone D was the most potent inhibitor against both α-glucosidases and free radicals. However, there are no previous reports on anti-tyrosinase activity of both compounds.

4.2 Effect of plant compounds on cell viability

To assess the effect of piperine, horsfieldone A and maingayone D on cell viability, B16F10 melanoma cells were used as *in vitro* model and kojic acid was used as assay control. Cells were cultured and treated with various concentrations of piperine, horsfieldone A, maingayone D or kojic acid. Cell viability was measured by using the MTT assay after 72 h treatment. MTT is a pale yellow compound that is converted by living cells to a dark blue formazan product. Since dead cells do not perform this reaction, MTT remains yellow in color at the end. Therefore, living cells will appear blue while dead cells appear yellow after treatment with MTT (Huang, Chiu & Chang, 2011). Results are expressed as the percent viability relative to control (0 µM). It can also be used to determine the cytotoxicity of potential medicinal agents and toxic materials, since those agents stimulate or inhibit cell viability and growth.

According to Figure 1, piperine exhibited cytotoxicity against B16F10 melanoma cells at concentration of 44 µM with a cell viability decreasing to approximately 80% of control after 72 h treatment. Horsfieldone A exhibited cytotoxicity against B16F10 melanoma cells at concentration of 11 µM with a cell viability decreasing to approximately 80% of control (Figure 2). Maingayone D showed toxicity at a concentration of 4 µM with a cell viability of 80% (Figure 3), whereas kojic acid did not have cytotoxic effects on the viability of B16F10 melanoma cells at concentrations lower than 880 µM (Figure 4). No visible changes in cell morphology were noted between the treated- and control groups at different concentrations.

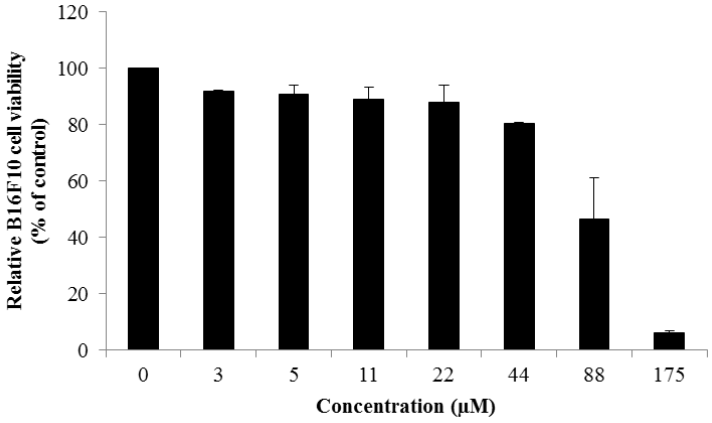

Figure 1 The effect of piperine on cell viability in B16F10 melanoma cells

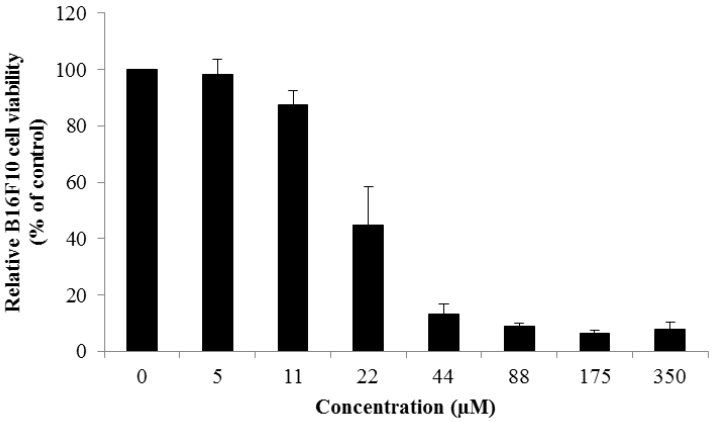

Figure 2 The effect of horsfieldone A on cell viability in B16F10 melanoma cells

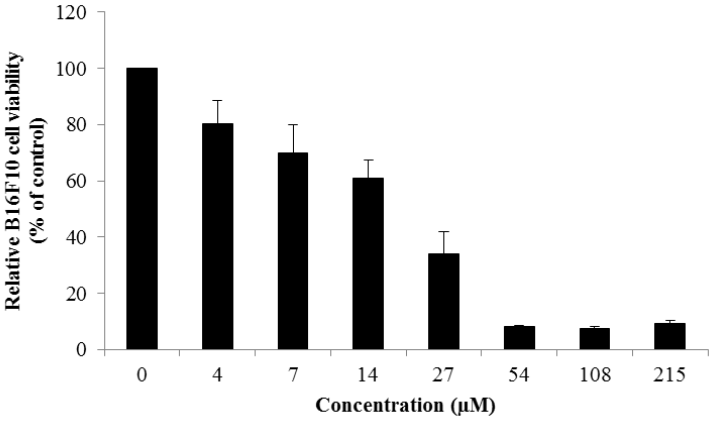

Figure 3 The effect of maingayone D on cell viability in B16F10 melanoma cells

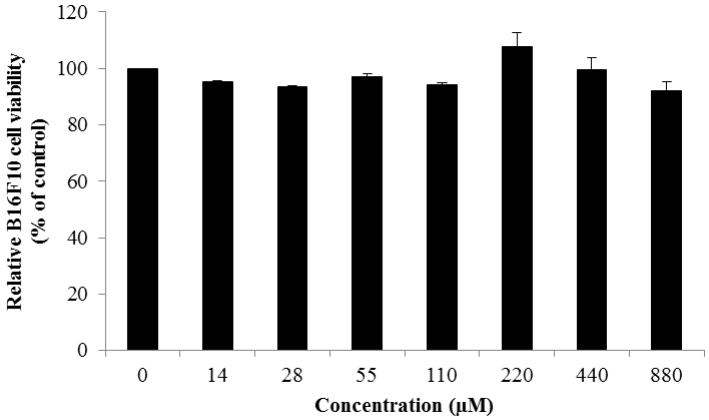

Figure 4 The effect of kojic acid (positive control) on cell viability in B16F10 melanoma cells

As in Table 3, it revealed that piperine, horsfieldone A and maingayone D were highly toxic with IC_{50} values of 79, 21 and 19 µM respectively. Compared to kojic acid (IC_{50} value of 10,765 µM), maingayone D showed the highest toxicity, while piperine and horsfieldone A were less toxic. These results were in agreement with a previous study which reported that piperine was non-toxic on B16F10 melanoma cells at concentrations ranging from 0 to 25 µM (IC_{50} value of 99 µM) (Heriniaina et al., 2018). Therefore, these compounds were not cytotoxic to B16 cells at their optimal concentrations. However, there are no previous reports on cell viability in horsfieldone A and maingayone D.

Table 3 The IC_{50} values of the most effective compounds on cell viability

Name of Compounds	Sources	IC_{50} value (µM)
Piperine	*Piper nigrum*	79
Horsfieldone A	*Horsfieldia motley*	21
Maingayone D	(*macrobotrys*)	19
Kojic acid		10,765

5. Conclusion

Fifteen compounds from six different plants were used to test for the anti-tyrosinase activity. The results demonstrate that piperine, horsfieldone A and maingayone D had high tyrosinase inhibition and also non-toxic to B16F10 melanoma cells. Furthermore, it is suggested that piperine, horsfieldone A and maingayone D are potential for tyrosinase inhibitors. They have the potential to be ingredients in cosmetic products for skin whitening. Further studies are necessary to investigate a mechanism of inhibitory of these three compounds.

6. Acknowledgements

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