Evaluation of Isolated Compounds from Wood of *Artocarpus heterophyllus* as A Cosmetic Agent

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Abstract

In our efforts to find new whitening agent materials, we focused on wood of *Artocarpus heterophyllus* which is anti melanogenesis. By activity-guided fractionation of *A. heterophyllus* wood extract, norartocarpetin and artocarpesin were isolated which inhibited both mushroom tyrosinase activity and melanin formation in B16 melanoma cells. This compound is a strong candidate as a remedy for hyperpigmentation in human skin that can be used for cosmetic (whitening agent).

Key words: tyrosinase, melanin, hyperpigmentation, wood, *Artocarpus heterphyllus*.

Introduction

Melanin pigments are formed in specialized pigment-producing cells known as melanocytes, which originate in the neural crest during embryogenesis and are distributed throughout the embryo during its development. At the cellular level, these compounds are biosynthesized in the membranous organelles known as melanosomes (Sánchez-Ferrer *et al*. 1995).

Cutaneous hyperpigmentation, including freckles, skin stains, and senile lentigines, is a common pigmentary disorder in humans that becomes more prominent with aging, especially in Asians. We have focused on the possibility of using natural resources as whitening agents for cosmetic products. An intensive search for a naturally occurring substance that would inhibit melanin pigmentation has been performed by Shimizu *et al*. (1998). Up to now, most research on the regulation of melanogenesis has focused on the factors affecting tyrosinase, which catalyzes the rate-limiting step of the melanin biosynthesis pathway, specifically, the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequently to DOPA quinone. Kojic acid (Garcia and Fulton 1996) and arbutin (Virador *et al*. 1999) are known as tyrosinase inhibitors and are used as skin-whitening cosmetics. The authors of several recent reports have noted that some growth factors or cytokines such as basic fibroblast growth factor (bFGF) (Halaban *et al*. 1988), endothelin-1 (ET-1) (Imokawa *et al*. 1995) and α-melanocyte-stimulating hormone (α-MSH) (Thody 1999) are secreted from ultra violet (UV)-irradiated keratinocytes or melanocytes and stimulate the proliferation or melanogenesis of human melanocytes. We concluded that we should examine the melanin biosynthesis effect using cultured cells to develop new whitening agents.

In Indonesia, several plants are used in a form of traditional medicine known as "Jamu." The uses of Jamu fall into four categories of medicine: health care, beauty care (cosmetics), tonics, and bodily protection (Soedarsono and Harini 2002). The use of traditional medicines has increased in recent years, and provides an interesting, largely unexplored source for the development of potential new drugs.

The *Artocarpus heterophyllus* plant has properties that remedy disease by supplying cooling, tonic, and pectorial relief (the pulp and seeds), by alleviating diarrhea and fever (the roots), by acting as a sedative in convulsion (the woods), by activating milk in women and animals and acting as an antisyphilic and vermifuge in humans (the leaves), and by relieving ulcers and wounds (the leaf ash) (Khan *et al*. 2003). Recently, we have reported that isoprenoid-substituted compounds from *A. heterophyllus* did not inhibit tyrosinase activity, but they showed inhibitory activity on melanin production in B16 melanoma cells with little or no cytotoxicity (Arung *et al*. 2006a). In the present study, we evaluated other isolated compounds from wood of *Artocarpus heterophyllus* on tyrosinase and melanin formation inhibition assay on B16 melanoma cells in order to identify potential depigmenting agents for skin-whitening cosmetics.

Materials and Methods

Reagents

NaOH, DMSO L-tyrosine and L-DOPA were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Mushroom tyrosinase (2870 units/mg) was purchased from Sigma Chemical Co. (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA) from Dojindo Co, (Kumamoto, Japan). A 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (St. Louis, MO), EMEM from Nissui Chemical Co (Osaka, Japan). Other chemicals are of the highest grade commercially available.

Isolation of Norartocarpetin and Artocarpesin

The wood meal of sapwood of *A. heterophyllus* (2.3 kg) was extracted with MeOH at room temperature repeatedly. The MeOH extract was concentrated *in vacuo*, gave a residue (60.6 g). A part of the extract (43.1 g) was

suspended in MeOH/H_2O (1:2) and partitioned with *n*-hexane, diethyl ether and EtOAc. The diethyl ether soluble (16.3 g), which showed potent inhibitory effect of melanin production in B16 melanoma cells, was applied to silica gel column (1633 g of Wakogel C-200, 11 x 90 cm) and eluted with *n*-hexane/EtOAc (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10 and MeOH, each 800 ml) to give sixteen fractions (Fr 1 to Fr 16). Fr 8 (2.04 g) was repeatedly chromatographed over silica gel (400 g of Wakogel C-200, 6 x 50 cm) and eluted with *n*-hexane/EtOAc (6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10, and 9:1, 7:3, 5:5, 3:7, 1:9, 0:10 of EtOAc/MeOH, each 600 ml and MeOH 1200 ml) to give nine fractions (Fr 8-1 to Fr 8-8). Fr 8-3 (0.9 g) was subjected to silica gel column (400 g of Wakogel C-200, 3 x 40 cm) and eluted with *n*-hexane/EtOAc (10:0 - 0:10, and 9:1 - 1:9 of EtOAc/MeOH, and 5:5, 3:7, 1:9, 0:10 of Acetone/MeOH each 50 ml) to give 50 fractions (Fr 8-3-1 to Fr 8-3-50). Fraction Fr 8-3-12 was subjected to preparative HPLC (Inertsil Prep-ODS:20 mm i.d. x 250 mm) eluting with MeOH/H_2O (0.1% trifluoroacetic acid, TFA), 20:80, 8 ml/min, to afford norartocarpetin **(**3.9 mg) and artocarpesin (3.7 mg).

The compound structures were recorded at 400 MHz on JNM-AL400 FT NMR spectrometer (Jeo1). All compounds were dissolved in DMSO-d_6, methanol-d_4, and chemical shifts were referred to deuterated solvents. The compounds were assigned for ^{1}H, ^{13}C, HMQC, and HMBC. The NMR data of norartocarpetin and artocarpesin were compared with the previous reports, Lin *et al*. (1995) and Zheng *et al*. (2008), respectively.

Tyrosinase Enzyme Assay

Although mushroom tyrosinase differs somewhat from other sources, this fungal source was used for the present experiment due to its ready availability. It should be noted that the commercial tyrosinase was reported to contain numerous proteins besides tyrosinase (Kumar and Flurkey 1991), but was used without purification. The tyrosinase activity was determined with method as previously described by Arung *et al*. (2006b). Briefly, all the samples were first dissolved in DMSO and used for the actual experiment at 30 times dilution. First, 333 μL of 800 μM L-DOPA solution was mixed with 600 μL of 0.1M phosphate buffer (Na_2HPO_4•$12H_2O$-NaH_2PO_4•$2H_2O$) (pH 6.5), and incubated at 25°C. Then, 33 μL of the sample solution and 33 μL of the aqueous solution of mushroom tyrosinase (1380 units/mL) was added to the mixture and immediately measured the initial rate of linear increase in optical density at 475 nm, on the basis of the formation of DOPAchrome in the case of using L-DOPA as a substrate. In the case of using L-tyrosine as a substrate, reaction solution was incubated at 25°C for 20 min and the absorbance at 475 nm was measured before and after incubation. The reaction was started by addition of the enzyme. Although tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, the assay was carried out in air-saturated solution. Controls, without inhibitor were routinely carried out. Each experiment was carried out in duplicate or triplicate, and replicate values were usually within 5% of each other. Kojic acid was used as a positive control.

Cell Culture

A mouse melanoma cell line, B16, was obtained from RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/ml theophylline. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Inhibitory Effect of Melanin Biosynthesis using Cultured B16 Melanoma Cells

This assay was determined as described by Arung *et al*. (2007). Briefly, confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed from the plastic using 0.25 % trypsin/EDTA. The cells were placed in two plates of 24-well plastic culture plates (1 plate is for determining of melanin and other is for cell viability) at a density of 1×10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media were replaced with 998 μl of fresh media and 2 μl of DMSO was added with or without (control) the test sample at various concentrations (n=3) and arbutin was used as a positive control. The cells were incubated for an additional 48 h, and then the medium was replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed (see below).

Determination of Melanin Content in B16 Melanoma Cells

The melanin content of the cells after treatment was determined as follows. After removing the medium and washing the cells with PBS, the cell pellet was dissolved in 1.0 ml of 1*N* NaOH. The crude cell extracts were assayed using a micro plate reader (Bio-Tek, USA) at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a percentage of the results from the control culture.

Cell Viability

Cell viability was determined by use of the micro culture tetrazolium technique (MTT). The MTT assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells. Culture was initiated in 24-well plates at 1×10^5 cells per well. After incubation, 50 μl of MTT reagent [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide in PBS (5 mg/ml)] was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37°C for 4 h. After the medium was removed, 1.0 ml isopropyl alcohol (containing 0.04 N HCl) was added into

the plate, and the absorbance was measured at 570 nm relative to 630 nm.

Results and Discussion

By organic solvent fractionation, silica gel column chromatography, and preparative HPLC of fraction 8 from MeOH extract of *A. heterophyllus*, two compounds were isolated, norartocarpetin and artocarpesin (Figure 1). We examined the effect of these compounds on tyrosinase inhibitory activity and melanin biosynthesis inhibition in B16 melanoma cells. Figure 2 showed tyrosinase inhibitory activity of norartocarpetin and artocarpesin at 1, 5, 25, 50 and 100 µg/ml, while kojic acid 100 µg/ml as a positive control. The 50% inhibition of concentration (IC_{50}) of norartocarpetin and artocarpesi were 0.5µg/ml (1.7 µM) and 3 µg/ml (8.5 µM), respectively. The IC_{50} Kojic acid was 2 µg/ml (14.1µM) of concentration. The inhibition of norartocarpetin is superior compare to artocarpesin and kojic acid. In the *in vitro* experiments, using B16 melanoma cells, norartocarpetin and artocarpesin were more potent at down-regulating melanin production in the cells than the positive controls, arbutin. In this study, we used arbutin and kojic acid as positive controls because they are used in cosmetic products (Cabanes *et al*. 1994; Virador *et al*. 1999; Curto *et al*. 1999). In the Figure 3, melanin inhibition of norartocarpetin and artocarpesin were dose dependently. Norartocarpetin inhibited 47% of melanin production without any cytotoxicity on the cells at 60 µg/ml. Artocarpesin at high concentration (50 µg/ml) showed inhibition on melanin production (82%) but caused cytotoxic effect on cell proliferation (71%). In lower consentration (12.5 µg/ml), artocarpesin depicted the inhibition of melanin production (40%) with less cytotoxicity effect (99%). Based on these results, it appears norartocarpetin and artocarpesin may be a candidate for practical use in the future as long as its safety is guaranteed. However, the ability of norartocarpetin and artocarpesin to reduce melanin biosynthesis in mushroom tyrosinase assay may be related to the presence of a 4-substituted resorcinol moiety in its chemical structure. As suggested by Shimizu *et al*. (2000), Jimenez and Carcia-Carmona (1997), and Chen *et al*. (2004) a 4-substituted resorcinol moiety inhibits tyrosinase activity. The presence of isoprenoid substituent in C-6 decreased its tyrosinase inhibition activity as showed by artocarpesin result. The presence of isoprenoid moiety in C-3 or C-6 position reduced their tyrosinase inhibition activity (Lee *et al*. 2004) but the presence of isoprenoid moiety increase its capability to inhibit melanin production (Arung *et al*. 2007).

The mechanism of inhibition of melanin production in B16 melanoma cells by norartocarpetin and artocarpesin are still unclear, but although we believe that a 4-substituted resorcinol moiety may be related to the inhibitory activity. Therefore, further experiments are needed in order to determine the exact mechanism of these compounds.

In conclusion, norartocarpetin and artocarpesin are a promising compound that could be useful for treating hyperpigmentation, as a skin-whitening agent. However, it should be noted that safety is a primary consideration for its practical use in humans.

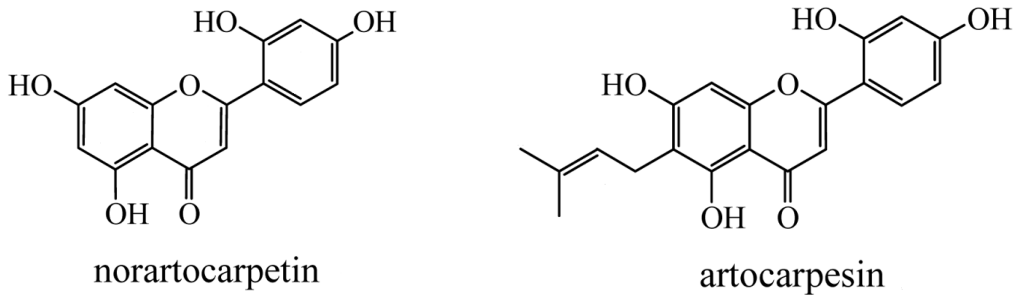

Figure 1. Structure of norartocarpetin and artocarpesin.

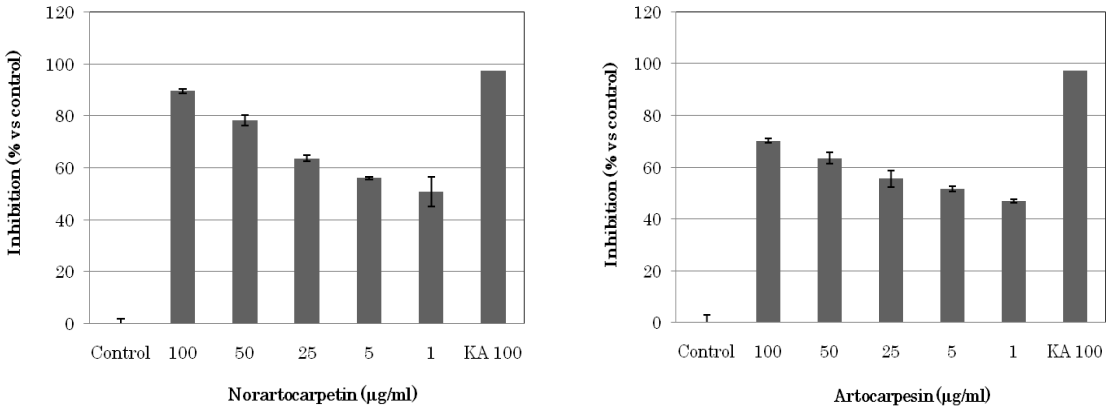

Figure 2. Tyrosinase inhibition activity of norartocarpetin and artocarpesin (KA100 : Kojic acid 100 μg/ml).

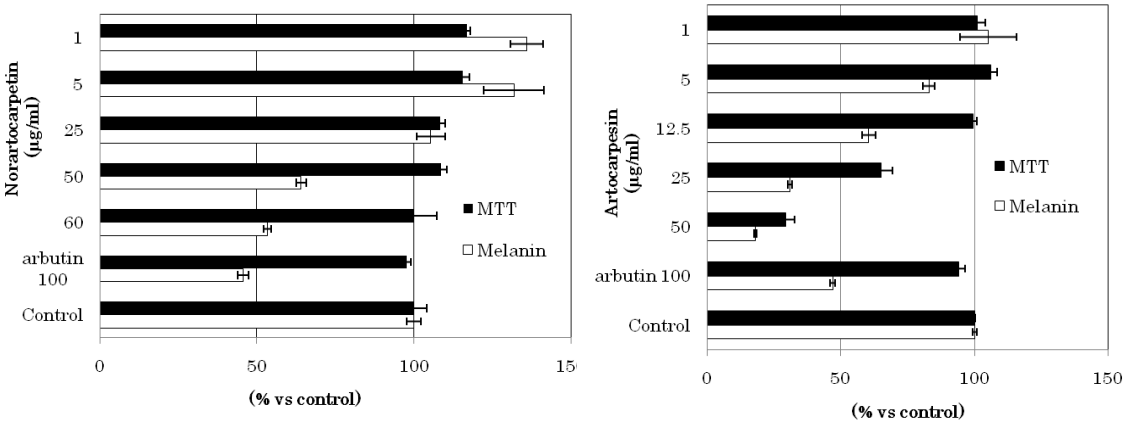

Figure 3. Melanin inhibition in B16 melanoma cells of norartocarpetin and artocarpesin (arbutin 100 : 100 μg/ml).

Acknowledgment

We thanked to Japan Society for Promotion of Science (JSPS) to support this experiment.

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