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Evaluation of the effect of Thai breadfruit's heartwood extract on melanogenesis-inhibitory and antioxidation activities

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Synopsis

The aim of this study was to clarify the melanogenesis-inhibitory and antioxidant activity of Thai breadfruit's heartwood extract for application as a skin-lightening agent. The heartwood of breadfruit (*Artocarpus incisus*) grown in Phitsanulok Province, Thailand, was extracted by using diethyl ether or methanol. The amount of artocarpin, a major component of *A. incisus* extract, was determined by using the HPLC method. The artocarpin content found in ether extract was 45.19 ± 0.45% w/w, whereas that in methanol extract was 19.61 ± 0.05% w/w. The ether extract was then evaluated for tyrosinase-inhibitory, melanogenesis-inhibitory, and antioxidant activities. The tyrosinase-inhibitory activity was tested *in vitro* by monitoring the inhibition of the extract against the formation of DOPAchrome by tyrosinase enzyme. The results showed that the tyrosinase-inhibitory activity of the extract was in a dose-dependent manner. The obtained IC_{50} value was 10.26 ± 3.04 μg/ml, while kojic acid, a well-known tyrosinase inhibitor, provided an IC_{50} of 7.89 ± 0.18 μg/ml. Melanocyte B16F1 melanoma cells (ATCC No. CRL-6323) were then used for determination of the melanogenesis-inhibitory activity of the extract, comparing it to hydroquinone, kojic acid, and purified artocarpin. The amount of melanin produced by the cells was monitored by measuring an absorbence at 490 nm. The obtained results indicated that *A. incisus* extract at a concentration of 2 to 25 μg/ml was able to decrease the melanin production of the melanocyte B16F1 cells. The obtained micrograph also confirmed that the extract did not change the cell morphology but reduced the melanin content by inhibiting melanin synthesis, whereas the purified artocarpin at a concentration of 4.5 μg/ml caused changes in cell morphology. Additionally, the extract exhibited antioxidant activity in a dose-dependent manner at an EC_{50} of 169.53 ± 9.73 μg/ml, according to DPPH assay. The obtained results indicated that the ether extract of *A. incisus*'s heartwood has the potential of acting as a skin-lightening agent for application in cosmetics.

INTRODUCTION

In the cosmetic industry, the demand for multifunctional products and their efficiency are the keys to trends in technology, innovations, and the cosmetic market. Skin-

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lightening products are widely used for cosmetic purposes. The ideal lightening agents should have a potent, rapid, and selective bleaching effect on hyperactivated melanocyte cells and carry no short- or long-term side effects.

Interference with the melanin synthesis of the lightening agents can be achieved by regulating the activity of melanogenic enzymes, the distribution of melanosomes, and the turnover of pigmented keratinocytes. Most lightening agents act specially to reduce the function of tyrosinase, which is the key enzyme in melanin biosynthesis (1). Nowadays, several researchers have reported that the lightening effects relate to antioxidant properties. Compounds with redox properties can have depigmenting effects by interacting with *o*-quinones and avoiding the oxidative polymerization of melanin intermediates. The phenolic derivatives of flavonoids are the most important antioxidants that can chelate the copper ions in tyrosinase. These indicate that the antioxidants can inhibit the activity of the tyrosinase enzyme and could be effective as lightening agents (1). Recently, safe and effective tyrosinase inhibitors extracted from natural sources have been reported for their potential applications in improving hyperpigmented disorders. For example, extracts from *Glycyrrhiza glabra* (licorice), *Morus alba* L. (white mulberry), *Carthanus tinctorius* L. (safflower), *Arctostaphylos Uva-Ursi* (bearberry), and *Oryza Sativa* (rice bran) have been used as skin-lightening agents. These materials are mostly free from harmful side effects. For this reason, there is an increasing interest in finding natural tyrosinase inhibitors from natural sources.

Artocarpus incisus (breadfruit) belongs to the Moraceae family. This evergreen tree, called "Sa-Ke" in Thai, is found throughout the tropical world. Because its pulp contains a high content of carbohydrate in the amount of 76.7%, it has been used as an important source of energy over the years (2). It has been reported that the methanol extract of the heartwood of *A. incisus* grown in Okinawa, Japan, strongly inhibits tyrosinase activity (3). Additionally, the mother liquor from crystallization of *A. incisus* ether extract also shows a melanin biosynthesis-inhibitory effect on brown guinea pigs. The heartwood extract of *A. incisus* consists of several flavonoids including artocarpin, (+)-norartocarpin, artocarpesin, (+)-dihydromorin, chlorophorin, (+)-norartocapanone, artocarbene, 4-prenyloxyresveratrol, and cycloartocarpin. Among these compounds, chlorophorin, (+)-norartocapanone, artocarbene and 4-prenyloxyresveratrol show much higher tyrosinase-inhibitory activity than kojic acid, whereas artocarpin does not show tyrosinase-inhibitory activity. However, this compound shows a skin-lightening effect on the UVB-induced hyperpigmented dorsal skin of brownish guinea pigs (4). It is likely that the *A. incisus* extract inhibits the melanogenesis process of melanocytes through other pathways. These findings lead to the question: does the crude extract exhibit higher melanogenesis-inhibitory activity than the purified artocarpin? Since the extract contains several kinds of flavonoids, the synergistic effects of tyrosinase-inhibitory, antioxidant, and other possible activities would be taking place.

Therefore, the aim of this research was to evaluate the potential of *A. incisus* crude extract for application as a skin-lightening agent. The heartwood of *A. incisus* was extracted by using various organic solvents, and the obtained extracts were then used to determine the amount of artocarpin, a major component of *A. incisus* extract, by using high-performance liquid chromatography (HPLC). The inhibitory effect of the extracts on melanin biosynthesis was investigated by using *in vitro* DOPAchrome assay and a cell culture model. Additionally, an *in vitro* DPPH assay was performed to determine the

antioxidant activity of the extracts. The obtained results indicate the potential of the new source for skin-lightening application.

MATERIALS AND METHODS

PLANT MATERIALS

The heartwood of *A. incisus* was collected from May to June of 2005 from Phitsanulok Province, Thailand. The heartwood was chipped in the size of 1×1×10 cm and exposed to the sun for two days. Then the chipped heartwoods were dried at 50°C for two days by using a hot-air oven and were ground into a powder by using a mill. The obtained powder was kept in a tight container at room temperature before being used.

EXTRACTION PROCESS

Two solvent systems, diethyl ether (analytical grade, Batch No. 04090204, LabScan Asia Co. Ltd., Bangkok, Thailand) or methanol (HPLC grade, Batch No. 03040021, LabScan Asia Co. Ltd.) were used for preparation of *A. incisus* extract with a modified method (3). According to our preliminary study, the different ratios between the amount of *A. incisus* powder and each solvent were selected due to the different capacity of each solvent to provide the highest percent yield of artocarpin in the extracts (8.25 g/1 kg for ether extract, 10.95 g/1 kg for methanol extract).

Five-hundred grams of the *A. incisus* powder was placed in a percolator and then soaked with 800 ml of diethyl ether at room temperature for two days. The solution of *A. incisus* ether extract was filtered through a woven cloth filter and evaporated to a concentrate under reduced pressure with a vacuum evaporator set at 30°C. For preparation of the methanol extract, one kilogram of *A. incisus* powder was placed in the glass container and then soaked with 2 l of methanol. The tight container containing the extracts was shaken at room temperature for one week. The solution of *A. incisus* extract was filtered and evaporated to a concentrate under reduced pressure with a vacuum evaporator at 50°C. After that, both kinds of extract were dried in a dessiccator. The dried extract was stored at −20°C in a tight amber glass before being used.

DETERMINATION OF ARTOCARPIN CONTENT

Isocratic high-performance liquid chromatography (HPLC) was used to determine the amount of artocarpin, a major component contained in the *A. Incisus*'s heartwood extract. Artocarpin standard (purified artocarpin) was kindly provided by Dr. Kuniyoshi Shimizu, Faculty of Agriculture, Kyushu University, Japan. The HPLC instrument consisted of an SPD-10M10AVP diode array detector and an SCL-10A central unit (Shimadzu Co., Ltd., Kyoto, Japan). A 5-µm Alltima C18 column of 250 × 4.60 mm diameter (Alltech Associates Inc., Illinois) was applied. The effluent consisted of a mixture of methanol:water (85:15). The flow rate of the effluent was 1 ml/min, and the volume of injection was 50 µl. Identification and quantification of the artocarpin was based on a peak area at 282 nm. All the experiments were performed in triplicate.

DETERMINATION OF TYROSINASE-INHIBITORY ACTIVITY

The tyrosinase-inhibitory activity of the ether crude extract was determined by spectrophotometry with a modified method (5,6). The sample solutions of *A. incisus* extract or a positive control, kojic acid (analytical grade, Lot No. 0832534, Sigma-Aldrich, Steinheim, Germany), were prepared by dissolving the crude extract or kojic acid in dimethylsulfoxide (DMSO, analytical grade, Lot No. 0320064, Sigma Chemical Co. Ltd., Missouri). For each concentration of the sample solution, fours well were designated as A, B, C, and D. Each contained a reaction mixture (180 µl) as follows:
(A) 20 µl of 426 units/ml of mushroom tyrosinase (analytical grade, Lot No. 023K7024, Sigma-Aldrich), 140 µl of 20 mM phosphate buffer (pH 6.8), and 20 µl of DMSO
(B) 160 µl of 20 mM phosphate buffer (pH 6.8) and 20 µl of DMSO
(C) 20 µl of 426 units/ml of mushroom tyrosinase solution, 140 µl of 20 mM phosphate buffer (pH 6.8), and 20 µl of the sample solution
(D) 160 µl of 20 mM phosphate buffer (pH 6.8) and 20 µl of the sample solution

The mixed solution was incubated at room temperature for 10 min, and then 20 µl of 0.85 mM 3,4-dihydroxyphenylalanine (L-DOPA, analytical grade, Lot No. 023K7024, Sigma-Aldrich) was added to each well. After incubation at 25°C for 20 min, an amount of DOPAchrome produced in each well was measured with an absorbence at 490 nm by using a Spectra Count® microplate reader (Perkin Elmer, Inc., Massachusetts).

Tyrosinase-inhibitory activity was calculated by using the following equation:

$$\%\text{Tyrosinase inhibition} = [(A–B) - (C–D)/(A–B)] \times 100$$

where A = an absorbance of the mixture well (A), B = an absorbence of the mixture well (B), C = an absorbence of the mixture well (C), and D = an absorbence of the mixture well (D).

IC_{50}, the 50% inhibition of tyrosinase activity was calculated as the concentration of test samples that inhibit 50% of tyrosinase activity under experimental conditions. This study was performed in triplicate.

DETERMINATION OF ANTIOXIDANT ACTIVITY

We used 2,2-diphenyl-1-picrylhydrazyl (DPPH, analytical grade, Lot No. 083K0830, Sigma-Aldrich) to measure the free-radical scavenging activity of the extract, comparing it to positive controls including butylhydroxytoluene (BHT, analytical grade, Lot No. 78H0689, Merck, Danstadt, Germany) and L-ascorbic acid (ACS reagent, Lot No. 60780, Riedel-deHaen, Seelze, Germany). The degree of DPPH decoloration indicated the scavenging efficiency of the added sample solution. This DPPH assay was performed in triplicate under a modified method (7,8).

The sample solutions of the tested samples including *A. incisus* ether extract, BHT, and L-ascorbic acid were prepared by dissolving each of them with DMSO, methanol, and deionized water, respectively. The reaction mixture consisted of 150 µl of DPPH (0.2 mM, stored at −20°C until use) and 75 µl of the sample solution. This sample solution was replaced with methanol, DMSO, or deionized water for use as a blank solution. The mixture was vortex-mixed for 15 sec and left to stand for 30 min. After incubation, the absorbence of the remaining DPPH was measured at a wavelength of 515 nm by using

the Spectra Count® microplate reader. The radical scavenging activity was calculated as a percentage of DPPH decoloration using the following equation:

$$\%\text{Radical scavenging activity} = [1-(A_S/A_B)] \times 100$$

where A_S = an absorbence of DPPH with the tested sample and A_B = an absorbence of DPPH without the tested sample.

EC_{50}, the equivalent concentration to give the 50% effect, was determined by log-probit analysis using six to ten different final concentrations of the samples.

DETERMINATION OF MELANOGENESIS-INHIBITORY ACTIVITY

B16-F1 (ATCC No. CRL-6323) mouse melanoma cells were purchased from the American Type Culture Collection (Lot No. 2634963, Virginia). This cell line was isolated from the melanocyte of mouse strain C57BL/6J. B16F1 melanoma cells were initially cultured in a 25-cm^2 flask (3.2×10^6 cells/ml) with DMEM (analytical grade, Lot No. 054K8302, Sigma-Aldrich, Missouri) supplemented with 10% fetal bovine serum (FBS, analytical grade, Lot No. 40f3634K, Gibco, Paisley, UK) in air containing 5% CO_2 and at a temperature of 37°C. The medium was changed every two days. The passage numbers of 5 to 8 were used in this study.

Before being tested, the cell suspension was transferred from the 25-cm^2 flask into a 24-well plate (1×10^5 cells/well) and kept in an incubator (5% CO_2 and temperature of 37°C) overnight for complete adherence of the cells on the plate. After 24 hr of cultivation, the old medium was replaced with 1.0 ml of new DMEM medium containing the various concentrations of *A. incisus* extract or artocarpin dissolved in DMSO. At the final concentration, the amount of DMSO used was not more than 0.1% (v/v). The control cells were treated with 0.1% (v/v) DMSO. Kojic acid and hydroquinone were used as positive controls in this study.

A melanin content assay was performed by using a modified method (9) with triplicate run. After treatment for three days, the treated cells were harvested by using trypsinization (Tyrpsin-EDTA, analytical grade, Lot No. 1212385, Gibco Canada Ltd., Ontario, Canada) and washed twice with phosphate buffer saline. The samples were air-dried and dissolved in 200 µl of 1 N NaOH containing 10% of DMSO (99.5% GC plant cell culture tested, CAS No. 67685, Merck, Shuchardt, Germany). The obtained solutions were heated at 80 °C for 1 hr and then cooled at room temperature. The absorbence of melanin was measured at a wavelength of 490 nm by using the Spectra Count® microplate reader. The melanin content per cell was calculated by comparing it to the absorbence of the control adjusted to 100%.

DETERMINATION OF CELL VIABILITY

Before being tested, B16F1 melanoma cells were initially cultured and treated as in the above procedure. After treatment for three days, the cell proliferation was measured by directly counting the number of cells treated with trypan blue. A hemocytometer was used for counting viable cells that were not stained with blue dye (trypan blue solution, R&D grade, Lot No. 55K2342, Sigma Chemical Co, Ltd.). The microscopic technique

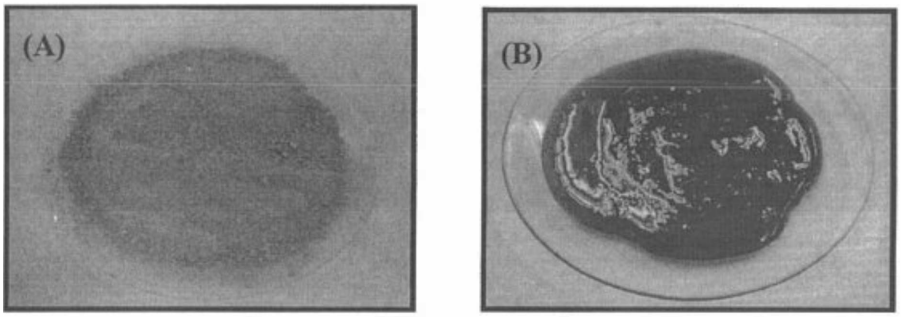


Figure 1. The appearance of *A. incisus* ether (A) and methanol (B) extract.

was used to investigate the phenotypic appearance of the melanocyte cells at, before, and after treatment with the tested samples.

STATISTICAL ANALYSIS

All experimental data were analyzed using analysis of variance (ANOVA), and the significant difference of the mean from triplicate analysis at $p < 0.05$ was determined by Duncan's multiple range test using SPSS 12.0 for Windows (SPSS Inc., Illinois).

RESULTS AND DISCUSSION

APPEARANCE AND ARTOCARPIN CONTENT OF THE EXTRACTS

The appearance, moisture content and percent yield of the diethyl ether or methanol extract are shown in Figure 1 and Table I.

Extraction of diethyl ether provided a yellow-powder solid, whereas that of methanol provided a deep brown paste. The HPLC chromatogram of artocarpin contained in both kinds of extract is shown in Figure 2. The amount of artocarpin contained in the ether and methanol extract was 45.19 ± 0.45 and 19.61 ± 0.05 % w/w, respectively. Because of the higher content of the major component, the ether extract of *A. incisus* was selected for further studies to clarify its action on melanogenesis-inhibitory and antioxidation activity.

Table I
Appearance, Percentage of Yield, and Moisture Content of Extracts from the Heartwood of *A. incisus*

	Type of extraction	
Characteristics	*A. incisus* ether extract	*A. incisus* methanol extract
% Yield	0.82	1.10
% Moisture content	7.19 ± 0.36	11.36 ± 0.78
Appearance	Yellow powder	Deep brown paste

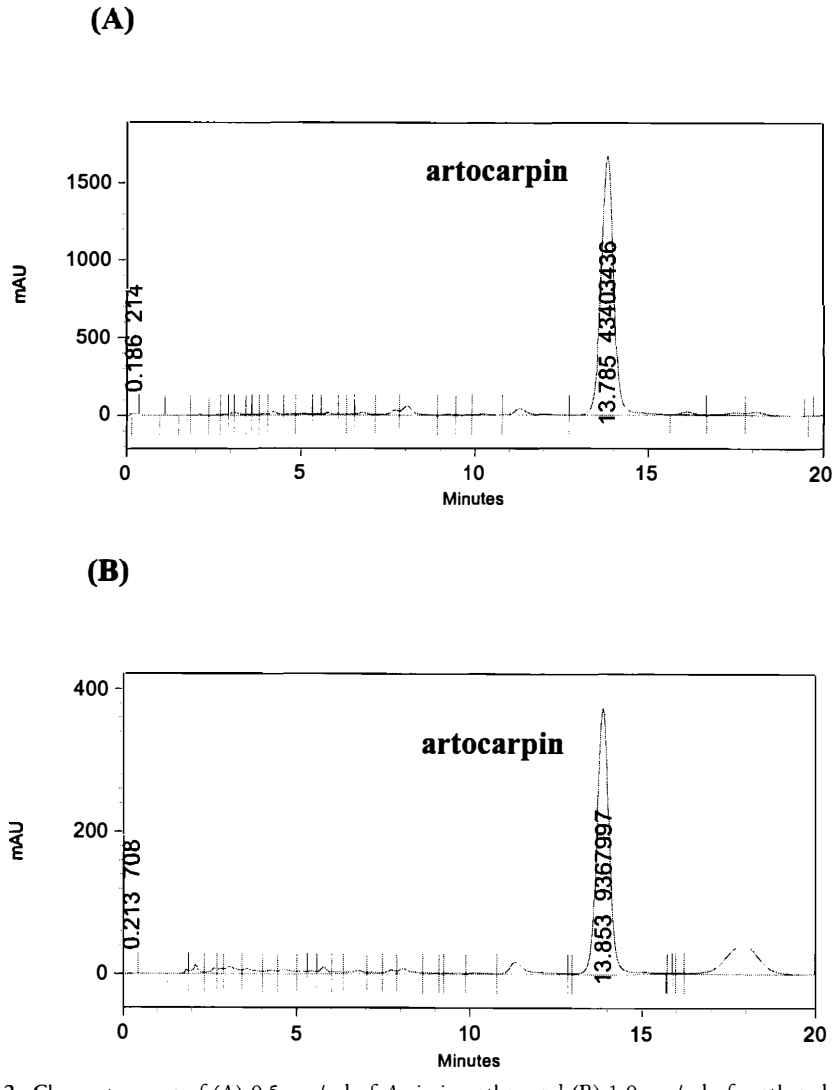


Figure 2. Chromatograms of (A) 0.5 mg/ml of *A. incisus* ether and (B) 1.0 mg/ml of methanol extracts.

TYROSINASE-INHIBITORY ACTIVITY

In this study, DMSO was used to dissolve the extracts. The percentage of tyrosinase inhibition of the ether extract and kojic acid are shown in Figure 3 and Table II. The IC_{50} value of the ether extract was 10.26 ± 3.04 μg/ml, whereas that of kojic acid, a well-known lightening agent, was 7.89 ± 0.18 μg/ml. Generally, the mode of inhibitory activity depends on the structure of both the substrate and inhibitor. In this study, such activity was concerned with the *o*-diphenolase inhibitory activity of mushroom tyrosinase since L-DOPA was used as the substrate (10,11). For this reason, similarly to kojic acid, the *A. incisus* extract behaves as an inhibitor of the diphenolase activity of tyrosinase. Previously, it was found that artocarpin showed no inhibitory effect, according to a mushroom tyrosinase assay (3,4). The tyrosinase-inhibitory effect of the *A. incisus* ether

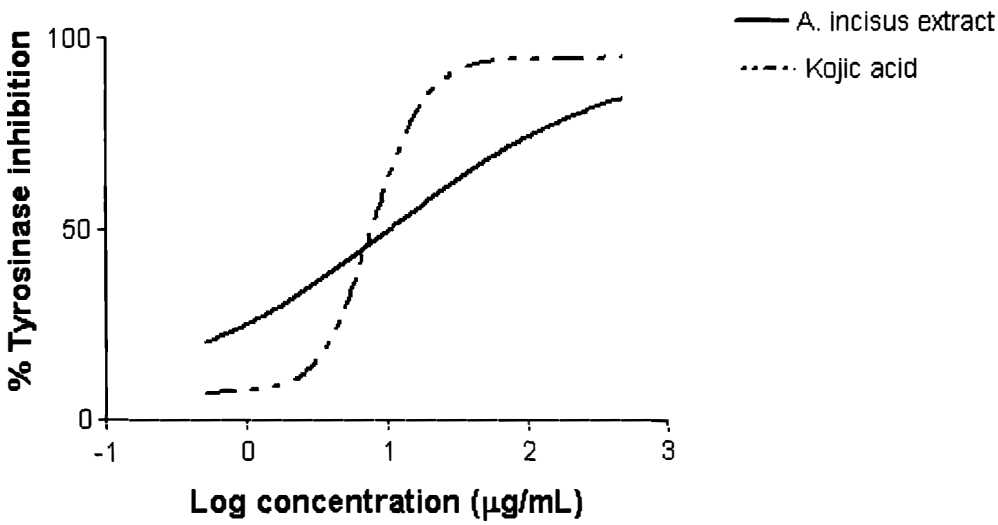


Figure 3. Tyrosinase-inhibitory activity of *A. incisus* ether extract and kojic acid.

Table II
Inhibitory Effects on Tyrosinase Activity of Kojic Acid and *A. incisus* Ether Extract

	Tyrosinase inhibition (%)	
Concentration (µg/ml)	Kojic acid	*A. incisus* ether extract
0.5	7.05 ± 0.08	20.13 ± 1.39
1	7.02 ± 0.04	26.55 ± 1.13
5	9.12 ± 0.11	42.76 ± 1.26
10	26.54 ± 2.33	48.29 ± 0.93
50	66.79 ± 0.53	67.44 ± 2.24
100	81.94 ± 3.16	73.40 ± 1.42
250	95.59 ± 0.55	81.25 ± 1.83
500	96.08 ± 0.95	85.06 ± 4.24
IC_{50} (µg/ml)	7.89 ± 0.18	10.26 ± 3.04

extract, therefore, may result from the action of the other compounds that are composed of such extract.

ANTIOXIDANT ACTIVITY

According to the DPPH radical scavenging activity, the EC_{50} values of *A. incisus* extract and the positive controls are presented in Table III and IV. Each tested sample shows the dose-dependent curve for DPPH radical scavenging activity, as shown in Figure 4. The obtained results indicate that the *A. incisus* ether extract has antioxidant activity with an EC_{50} of 169 ± 9.73 µg/ml. The extract of *A. incisus* heartwood provides weaker free-radical scavenging activity than BHT and L-ascorbic acid.

In this study, the radical scavenging activity of the ether extract was tested using a methanolic solution of the stable free redical, DPPH. This solution exhibits a deep purple color with an absorption maximum at 515 nm. Antioxidant molecules can quench DPPH free radicals and convert them to a colorless/bleached product. The

Table III
Percentage of Free-Radical Scavenging of *A. incisus* Ether Extract

Concentration of *A. incisus* ether extract (μg/ml)	Free radical scavenging (%)	EC_{50} (μg/ml)
0.5	3.64 ± 0.29	169.53 ± 9.73
1	7.70 ± 0.30	
5	10.58 ± 0.43	
10	13.09 ± 0.33	
25	15.35 ± 0.22	
50	20.30 ± 1.27	
100	41.67 ± 1.81	
250	62.71 ± 1.34	
500	77.89 ± 1.23	
2000	94.08 ± 0.92	

Table IV
Percentage of Free-Radical Scavenging of L-Ascorbic Acid and Butylated Hydroxytoluene

	Free radical scavenging (%)	
Concentration (μg/ml)	L-ascorbic acid	Butylated hydroxytoluene
0.25	7.83 ± 1.72	12.68 ± 0.76
0.5	15.81 ± 0.53	15.17 ± 1.64
2.5	41.02 ± 3.97	56.68 ± 0.91
5	69.03 ± 0.16	80.51 ± 0.73
25	88.10 ± 0.33	92.41 ± 1.41
50	93.89 ± 0.12	94.88 ± 0.23
125	94.01 ± 0.13	95.00 ± 0.08
250	94.09 ± 0.15	97.02 ± 1.35
1000	93.94 ± 0.13	96.12 ± 0.11
2500	93.96 ± 0.38	94.93 ± 1.46
EC_{50} (μg/ml)	3.16 ± 0.26	2.30 ± 0.03

DPPH test only recognizes free-radical scavenging effects and not pro-oxidant activity (8). Therefore, other antioxidant properties, such as reducing *o*-quinones or other intermediates in melanin biosynthesis of the ether extract, should be taken into consideration and further clarified in the future.

MELANOGENESIS-INHIBITORY ACTIVITY

In this study, the activity of melanocyte B16F1 cells was investigated by determination of the melanin content of cells treated with *A. incisus* ether extract, purified artocarpin, or lightening agents including kojic acid and hydroquinone. The *A. incisus* extract showed the dose-dependent inhibition of melanin production of B16F1 cells, as shown in Figure 5. The percentages of melanin reduction in the extract-treated cells compared to that of the control (DMSO-treated) cells at concentrations of 2, 10, 15, and 25 μg/ml of *A. incisus* extract were 5.34%, 8.76%, 16.73%, and 26.65%, respectively. The final concentration of DMSO in the cultured medium was not more than 0.1% v/v.

To provide more evidence that the extract of *A. incisus* potentially inhibits melanogenesis, we determined its effects on melanin production in comparison to well-known

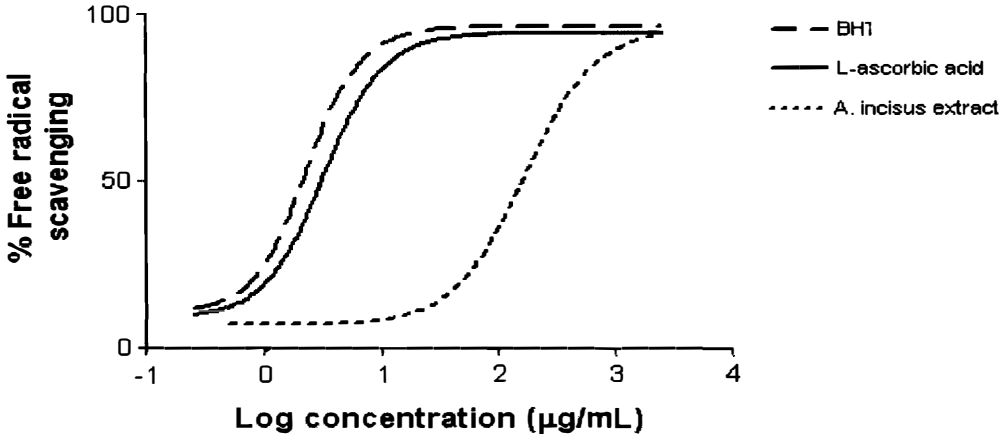


Figure 4. Antioxidant activity of *A. incisus* ether extract, L-ascorbic acid, and butylated hydroxytoluene.

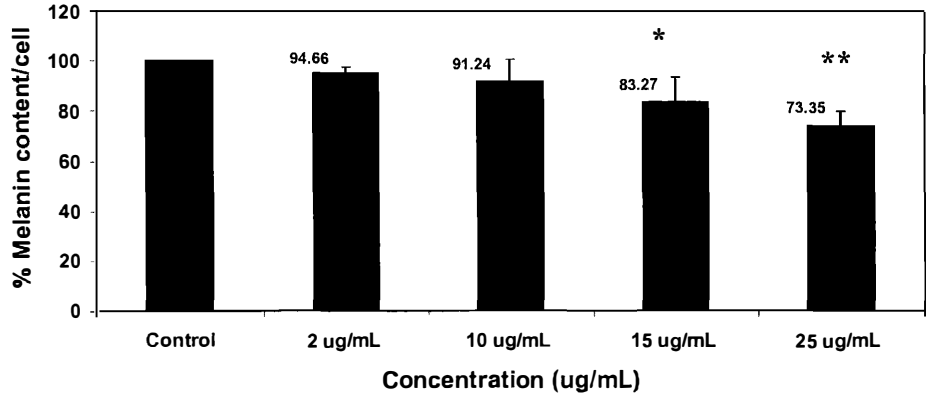


Figure 5. Inhibitory effects of *A. incisus* ether extract on melanin synthesis in melanocyte B16F1 melanoma cells. Data are expressed as percent of control, and each column represents mean ± S.D. of triplicate study. Significantly different from the control value: $*p < 0.5$, $**p < 0.01$.

skin-lightening agents, kojic acid and hydroquinone. The concentration of each tested sample used was 10 µg/ml, which corresponds to the IC_{50} of the ether extract, according to a previous study of tyrosinase-inhibitory activity. Additionally, purified artocarpin, the major component of the *A. incisus*'s heartwood extract, was investigated for its melanogenesis-inhibitory effect. Its concentration used was 4.5 µg/ml, which is equivalent to 4.5 µg/ml of artocarpin contained in the extract, according to a previous study of artocarpin content in the extract.

The effect of the tested samples on the melanin production of melanocyte B16F1 cells is shown in Figure 6. The reduction of melanin synthesis was expressed by kojic acid, *A. incisus* ether extract, artocarpin, and hydroquinone as 9.61%, 8.76%, 8.88%, and 40.97%, respectively, compared to the control cells. These results reveal that, at a similar artocarpin concentration, the inhibition of melanin production of the ether extract shows no difference from that of the artocarpin. Compared to kojic acid, it is likely that the potential of melanin reduction of the ether extract is close to that of kojic acid.

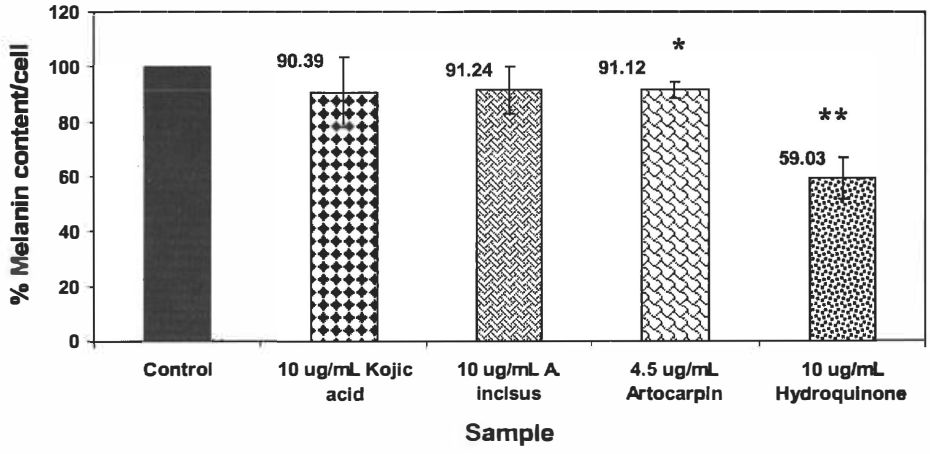


Figure 6. Effects of *A. incisus* ether extract, hydroquinone, kojic acid, and artocarpin on melanin production of melanocyte B16F1 melanoma cells. Data are expressed as percent of control, and each column represents mean ± S.D. of triplicate study. Significantly different from the control value: $*p < 0.5$, $**p < 0.01$.

Generally, skin pigmentation is determined by melanin synthesis within melanosomes and their distribution to keratinocytes within the epidermal melanin unit. Focusing on melanin synthesis, the rate-limiting step is the hydroxylation of tyrosine to DOPA by the enzyme tyrosinase. Since melanin production requires tyrosine, some mechanism, such as transportation of this amino acid through the melanosomal membrane, may be also a critical factor in limiting pigment production (12,13). As we know, kojic acid, a g-pyrone derivative, can strongly inhibit tyrosinase activity by chelation of the copper ion(s) at the active center of the enzyme (14,15). For artocarpin, as mentioned above, its tyrosinase-inhibitory activity could not be observed. This probably results from its steric hindrance structure since a 4-substituted resorcinol skeleton, coupled with a C3 substituent, is present in the flavone structure of artocarpin (16). Therefore, the melanogenesis-inhibitory activity of artocarpin may mainly involve the transportation of tyrosine through the melanosomal membrane, a subject that we need to clarify in the future.

CELL VIABILITY

As an attempt to find out if the reduction of melanin content in B16F1 cell treated with the ether extract causes the reduction of the viable cells, the determination of the effect of such an extract on cell proliferation was performed. B16F1 cells were treated with various concentrations (10, 25, and 100 µg/ml) of *A. incisus* ether extract for three days, and the number of cells was then determined. The numbers of viable cells were evaluated by staining cells with blue dye. As shown in Figure 7, *A. incisus* extract induced the dose-dependent inhibition of cell growth in B16F1 cells. The inhibitory effects on B16F1 cells were less profound at a low dose (<25 µg/ml) of the extract, whereas significant arrest of cell growth was observed at a concentration of 100 µg/ml. High doses of the extract may cause cell apoptosis and/or alteration of cell attachment, which is an important characteristic for the proliferation of adherent cells.

The effect of kojic acid, *A. incisus* extract, artocarpin, and hydroquinone on the proliferation of melanocyte B16F1 cells is shown in Figure 8. The obtained results indicate

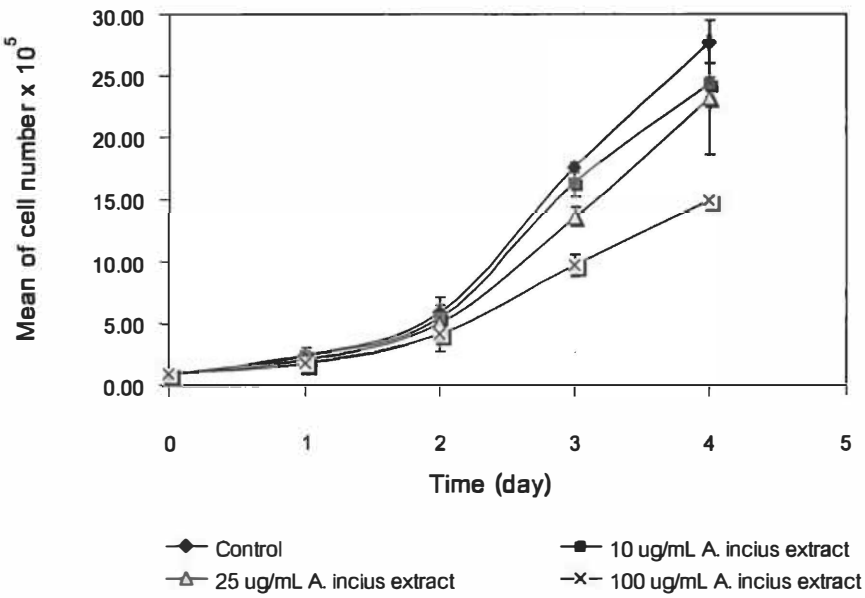


Figure 7. Effect of *A. incisus* ether extract on viability of melanocyte B16F1 melanoma cells. Each point represents mean ± S.D. of triplicate study.

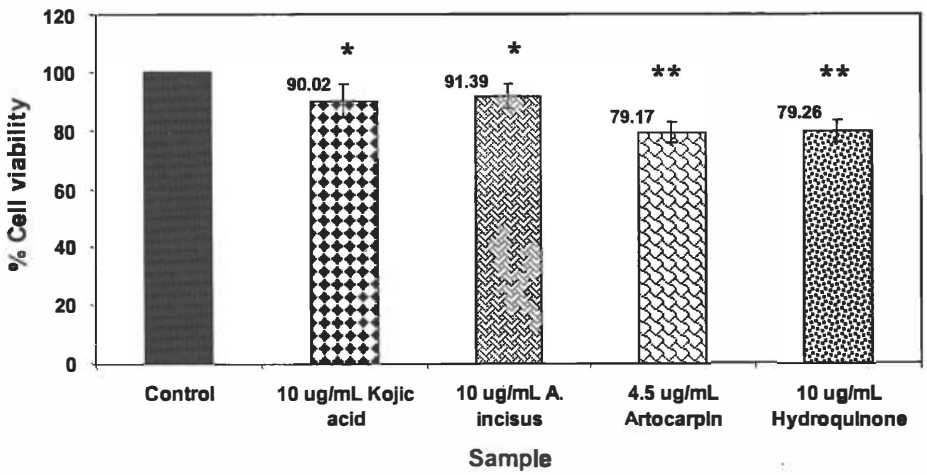
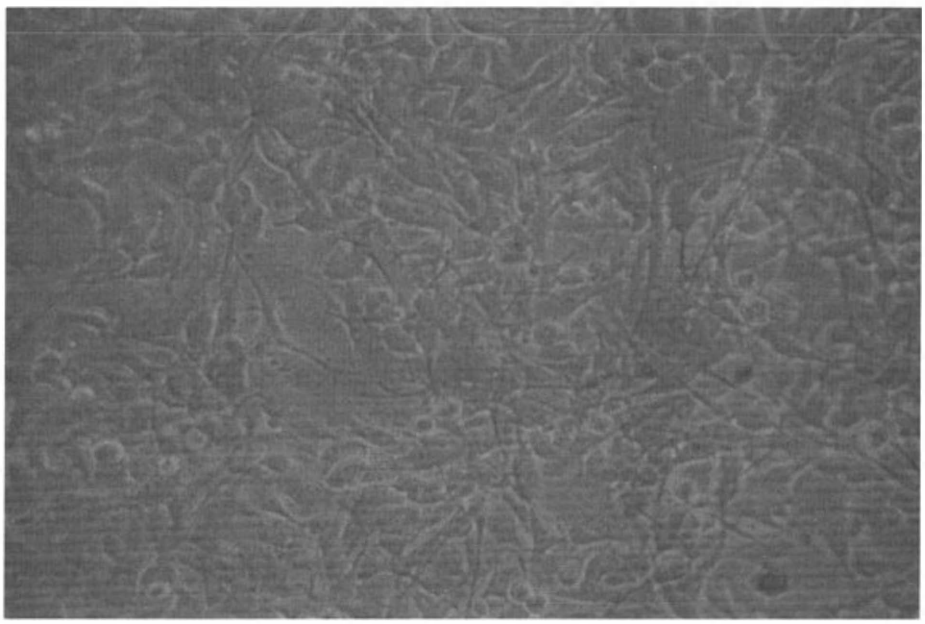


Figure 8. Effects of *A. incisus* ether extract, hydroquinone, kojic acid, and artocarpin on viability of melanocyte B16F1 melanoma cells. Data are expressed as percent of control, and each column represents mean ± S.D. of triplicate study. Significantly different from the control value: $*p < 0.5$, $**p < 0.01$.

that the percent viability of cells after being treated with kojic acid, *A. incisus* extract, artocarpin, and hydroquinone for three days was 90.02%, 91.39%, 79.17%, and 79.26%, respectively, as compared to the control cells. The microscopic technique was also used to investigate the phenotypic appearance of melanocyte cells to confirm the effect of these agents on melanocyte morphology. As shown in Figures 8 and 9, the number of cells markedly decreased after being treated with hydroquinone. Cell damage may result in a decrease in the production of melanin. Additionally, the dendrite

(A)



(B)

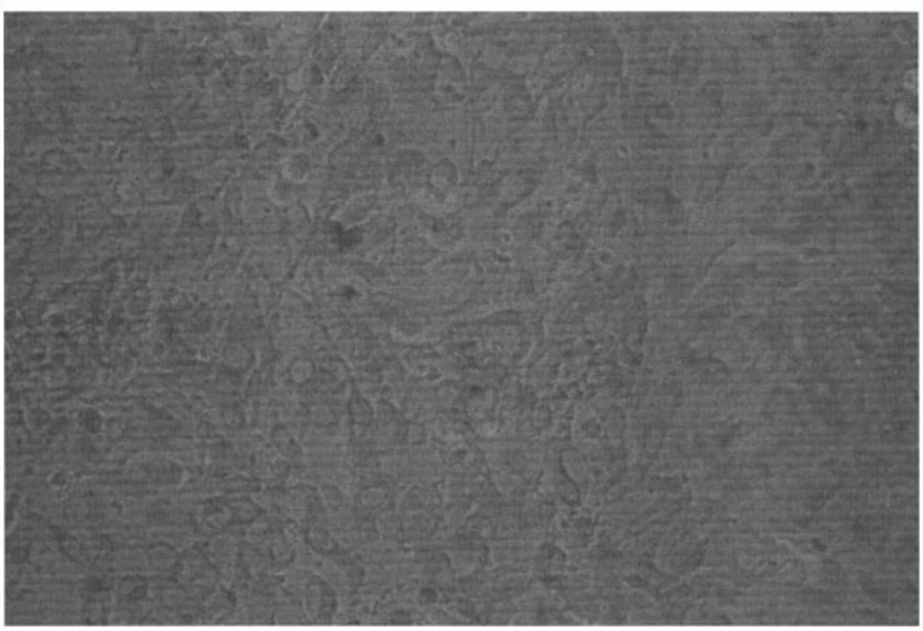


Figure 9. Morphology of melanocyte B16F1 melanoma cells treated with (A) 0.1% DMSO (control) and (B) 10 μg/ml of hydroquinone for three days. Magnification: 400×.

morphology of melanocyte B16F1 melanoma cells was shorter in length compared to the control cells. These obtained results indicate the cytotoxicity of hydroquinone at the concentration of 10 μg/ml used in this study, which was similar to the effect observed previously (17,18).

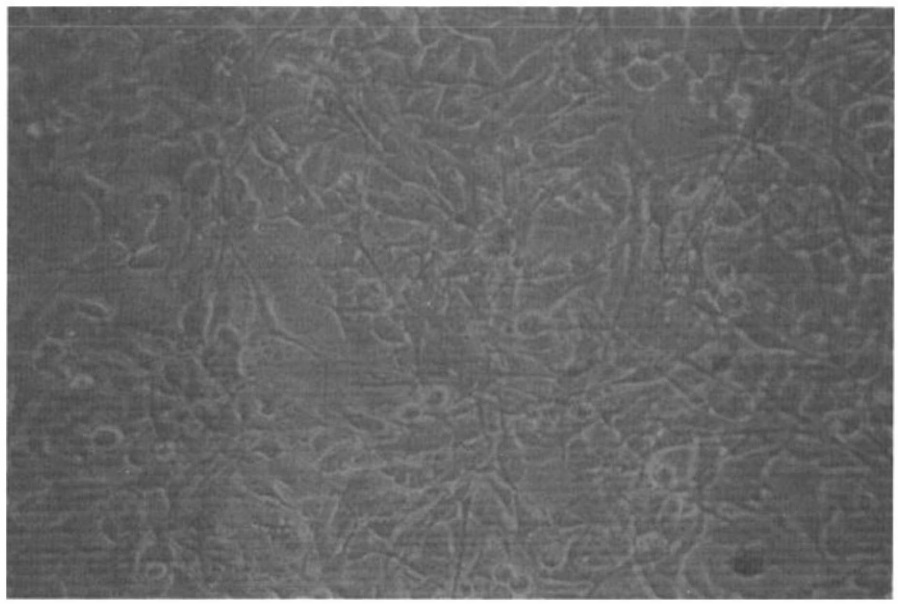
At a concentration of 10 μg/ml, kojic acid did not show a significant inhibitory effect on melanin production compared to the control as shown in Figure 6. Although kojic acid showed stronger tyrosinase inhibition than *A. incisus* extract based on the IC_{50} values from the DOPAchrom assay (IC_{50} values of kojic acid and the extract were 7.89 and 10.26 μg/ml, respectively), kojic acid showed lower inhibitory activity on melanogenesis than the ether extract in the cell culture model. These results correspond with those from the previous study that indicated a low- or non-inhibitory effect of kojic acid on the production of melanin in Melan-a, and MelAb cells (18–20). The lipophilic system of cell membrane may decrease the penetration of kojic acid through the cell, resulting in lower inhibitory action on the intracellular tyrosinase enzyme. In addition, as shown in Figure 10, we found changes in the morphology of the kojic acid-treated cells, in losing dendritics, according to the microscopic observation.

The effects of 10 μg/ml of *A. incisus* ether extract and 4.5 μg/ml of artocarpin on the proliferation and morphology of melanocyte cells are shown in Figures 8 and 11. We found that artocarpin significantly decreased the proliferation of cells, whereas the extract did not affect cell proliferation. The obtained micrographs of cells treated with artocarpin indicated more evidence of the effect of artocarpin on melanocyte morphology by losing dendrites but not destroying the cells. Furthermore, a higher concentration of artocarpin ($\geq$4.5 μg/ml) caused cell death. Surprisingly, *A. incisus* crude extract containing a similar artocarpin amount did not show cytoxicity in the melanocyte cells. Additionally, at higher concentration, the morphology of cells treated with the extract was not changed. This indicates that the extract can suppress melanin production without affecting cell morphology or cell growth. It is possible that the extract contains some components that are contributing to cell structure stabilization and/or protecting the cell from damage in response to external stimuli.

CONCLUSIONS

Nowadays the botanical extract plays an increasingly important role in cosmetics. In the research for new natural lightening agents, plant extracts with depigmenting effects, including tyrosinase-inhibitory activity, melanogenesis-inhibitory activity, and antioxidant activities have been investigated. As applied to cosmetics, isolation and purification of the active ingredient from the crude extract are sometimes not needed because such isolation and purification may lead to a loss of the biological activity and may lead to toxicity. Therefore, in this study, the crude extract of *A. incisus* was clarified in its melanogenesis-inhibitory and antioxidant activities. In the past, some studies reported the constituents and effects of *A. incisus*'s heartwood methanol extract on melanogenesis inhibition. However, as far as we know, there has never been a report on the effects of the ether extract of *A. incisus*'s heartwood on tyrosinase enzyme, melanogenesis, or oxidation activities.

(A)



(B)

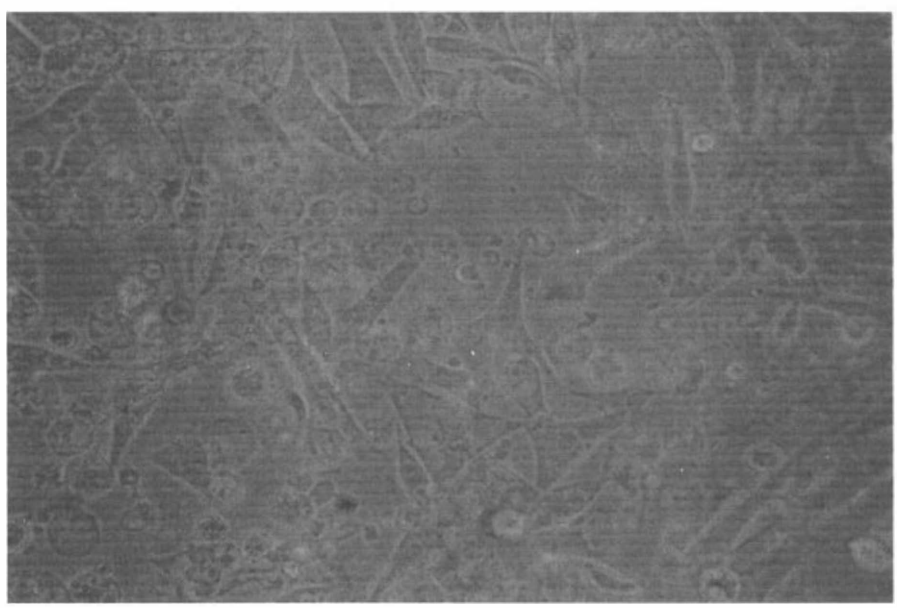
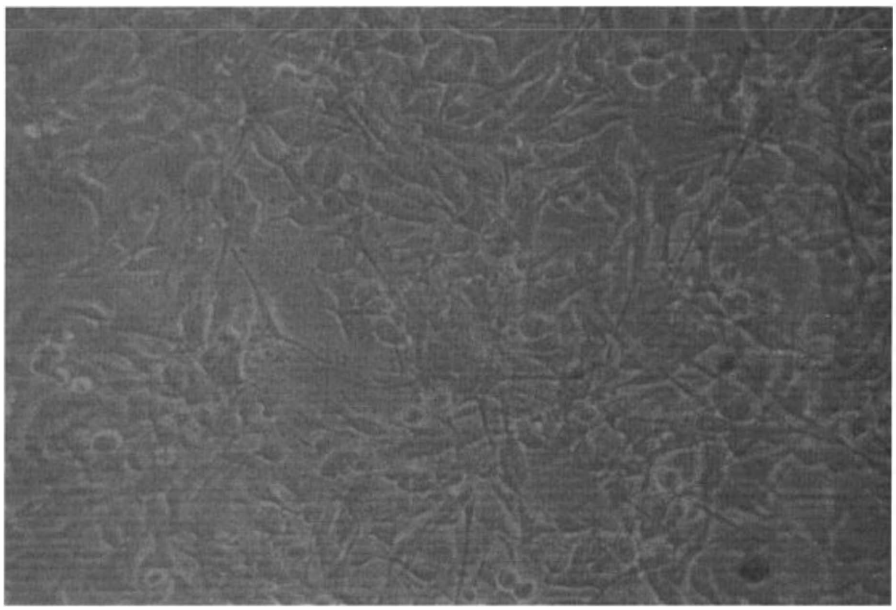


Figure 10. Morphology of melanocyte B16F1 melanoma cells treated with (A) 0.1% DMSO (control) and (B) 10 μg/ml of kojic acid for three days. Magnification: 400×.

(A)



(B)

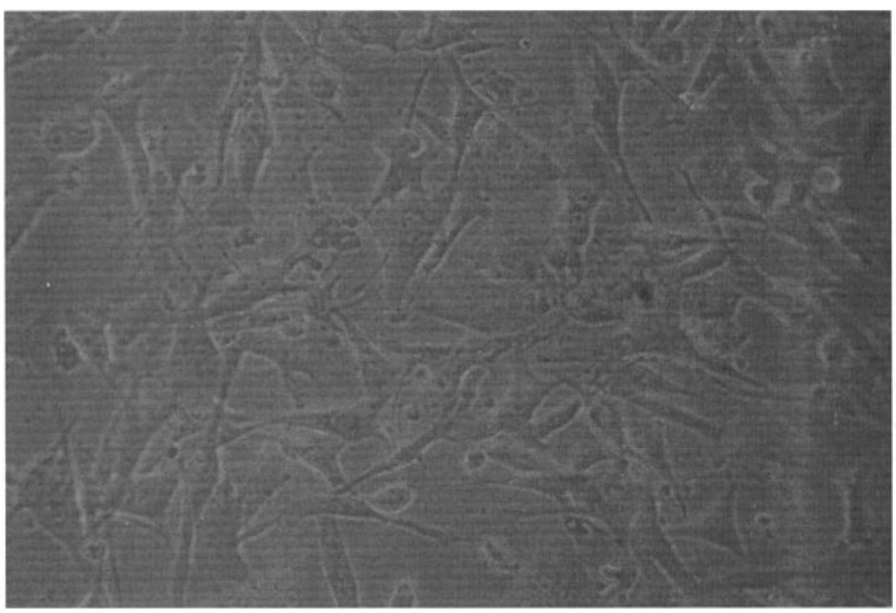


Figure 11. Morphology of melanocyte B16F1 melanoma cells treated with (A) 0.1% DMSO (control), (B) 10 μg/ml of *A. incisus* ether extract, and (C) 4.5 μg/ml of artocarpin for three days. Magnification: 400×.

(C)

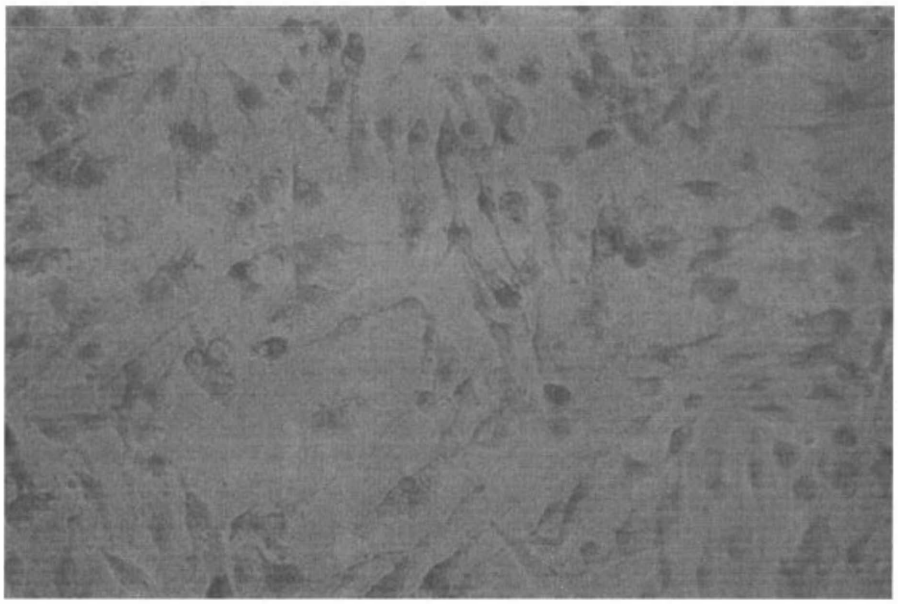


Figure 11. Continued

With all findings taken together, we have found that *A. incisus* crude extract of diethyl ether at a concentration of 10 μg/ml exhibits non-cytotoxicity, potent tyrosinase inhibitory activity, and the ability to reduce the melanin content in melanocyte cell culture. Previous studies revealed several kinds of phenolic compounds in the heartwood of *A. incisus.* Such phenolic compounds have been reported to have tyrosinase-inhibitory activity and have been studied as depigmenting agents (1,21). Additionally, their antioxidant property may prevent or delay pigmentation by different mechanisms, such as by scavenging ROS and reactive nitrogen species, or by reducing *o*-quinones or other intermediates in melanin biosynthesis. Therefore, the action of *A. incisus* ether extract on tyrosinase may be due, at least in part, to the phenolic components of the extract. Its antioxidant activity, together with its tyrosinase-inhibitory activity, would provide much more benefit. As compared to *Morus alba* (mulberry extract), the *A. incisus* ether extract seems to be a stronger tyrosinase inhibitor. IC_{50} tyrosinase inhibition of mulberry extract was 78.3 μg/ml, and EC_{50} DPPH radical scavenging activity was more than 100 μg/ml (22). Our findings indicate the potential of the *A. incisus* ether extract for application in skin lightening. However, further studies, including those on toxicity to the human cell, irritability, and *in vivo* toxicity and efficacy, should be performed to estimate the value of the extract for marketing in the future.

ACKNOWLEDGMENTS

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