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ARTICLE

Inhibitory Effect of Phlorotannins Isolated from *Ecklonia cava* on Mushroom Tyrosinase Activity and Melanin Formation in Mouse B16F10 Melanoma Cells

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In this study, to assess the feasibility of phlorotannins isolated from *Ecklonia cava* as an inhibitor of melanin formation, we evaluated its inhibitory effects on mushroom tyrosinase and 3-isobutyl-1-methylxanthine (IBMX)-induced melanin formation inhibitory effects in B16F10 melanoma cell. The ethanolic (EtOH) extract and ethyl acetate (EtOAc) soluble fraction obtained from *E. cava* evidenced a marked inhibitory effect on mushroom tyrosinase at a concentration of 50 μg/mL. Repeated column chromatography of the active EtOAc fraction resulted in the isolation of three phlorotannins. Their structures were elucidated on the basis of spectroscopic techniques [1D and 2D nuclear magnetic resonance (NMR)] and characterized as phloroglucinol (**1**), dioxinodehydroeckol (**2**), and 7-phloroeckol (**3**), respectively. Among the compounds, 7-phloroeckol (**3**) evidenced more potent tyrosinase inhibitory effect with an IC_{50} value of 0.85 μM than arbutin (IC_{50} = 243.16 μM) and kojic acid (IC_{50} = 40.28 μM), which were used as positive controls. Lineweaver−Burk plots suggest that 7-phloroeckol plays as a noncompetitive inhibitor against tyrosinase. Furthermore, these compounds were evaluated for their inhibitory effects on IBMX-induced melanin formation in B16F10 melanoma cells. Treatment with 7-phloroeckol (6.25−100 μM) resulted in a significant inhibition of melanin production in the melanoma cells. In this study, we suggest that 7-phloroeckol might prove useful as a novel inhibitor of melanin formation in cosmetic applications.

KEYWORDS: ***Ecklonia cava*; phlorotannins; 7-phloroeckol; tyrosinase; B16F10 melanoma cell**

INTRODUCTION

Melanin is essential for protecting human skin against radiation, but the accumulation of abnormal melanin induces pigmentation disorders, such as melasma, freckles, ephelide, and senile lentigines (*1*, *2*). Melanogenesis is conducted in the melanocytes, located in the basal layer of the epidermis and controlled by tyrosinase (*3*). Tyrosinase (EC 1.14.18.1), which is also referred to as polyphenol oxidase (PPO), is a copper-containing monooxygenase that is considered to be a key enzyme in melanin synthesis (*4*, *5*). Tyrosinase catalyzes two oxdative reactions: the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (DOPA), which is followed by the oxidation of DOPA to DOPA quinone (EC 1.10.3.1, catechol oxidase) (*6*). Quinones, highly reactive compounds, react with amino acids and proteins and form high-molecular-weight compounds or brown pigments (eumelanin or pheomelanin) that enhance the production of brown pigments (*7*). In an effort to inhibit the activity of tyrosinase, many different types of tyrosinase inhibitors have been developed via either synthesis or isolation from natural sources (*8*, *9*). These compounds are applicable for cosmetics as skin-whitening agents and also as drugs for use in the treatment of pigmentation disturbances (*10*, *11*). Despite the enormous amount of research conducted thus far into the development of new whitening agents, the use of existing agents is rather limited, owing to high toxicity, low stability, poor skin penetration, and insufficient activity (*12*). Because of the side effects of synthetic tyrosinase inhibitors, the development of novel tyrosinase inhibitors from natural products continues to arouse great interest.

Ecklonia cava Kjellman is a perennial brown alga belonging to the family Laminariaceae. This species is distributed widely throughout the coastal areas of Jeju Island, Korea. It has been used as a foodstuff, along with *Laminaria japonica* and *Undaria pinnatifida*. In previous studies, *E. cava* has been demonstrated to exhibit many beneficial bioactivities, including antioxidant, anticancer, anticoagulant, and matrix metalloproteinase inhibitory activities (*13*−*17*).

Phlorotannins, major metabolites of *Ecklonia* species, are polyphenolic compounds and have been shown to be responsible for a variety of positive physiological effects, including reactive oxygen species (ROS) scavenging, as well as angiotensin-converting enzyme and cholinesterase inhibitory activities (*18*−*20*).

In the present study, in an effort to develop a safe and effective melanin formation inhibitor, we have investigated

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the inhibitory effects of phlorotannins isolated from *E. cava* on mushroom tyrosinase activity and melanin formation in B16F10 melanoma cells.

MATERIALS AND METHODS

General Procedures. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were determined on a JNM ECP-400 spectrometer (JEOL, Tokyo, Japan) using DMSO-d_6 with tetramethylsilane (TMS) as an internal standard. Column chromatography was conducted with silica gel 60 (70−230 mesh, Merck, Darmstadt, Germany), RP-18 Lichroprep (Merck, Darmstadt, Germany), and Sephadex LH-20 (Sigma, St. Louis, MO). TLC was conducted on a precoated Merck Kieselgel 60 F_{254} plate (0.25 mm) and a RP-18 F_{254s} plate (Merck, Darmstadt, Germany), and the spots were detected under UV light using 50% H_2SO_4 reagent. All of the solvents for column chromatography were of reagent grade and were acquired from commercial sources.

Chemicals. Arbutin, kojic acid, 3-isobutyl-1-methylxanthine (IBMX), and mushroom tyrosinase (EC 1.14.18.1) were obtained from Sigma Chemical Company (St. Louis, MO). L-tyrosine and K_2HPO_4 were obtained from Junsei Chemical Co. Ltd. (Tokyo, Japan), and KH_2PO_4 was obtained from Yakuri Pure Chemicals Co. Ltd. (Osaka, Japan).

Plant Material. Leafy thalli of *E. cava* were collected along the coastal areas of Jeju Island, South Korea, between October 2004 and March 2005. A voucher specimen was deposited in the author's laboratory.

Preparation of Ethanolic Extract and Fractions. The lyophilized powder (4.0 kg) of *E. cava* was percolated in hot EtOH (3 × 10 L). The extract (584.3 g) was partitioned with organic solvents to yield *n*-hexane (114.3 g), dichloromethane (CH_2Cl_2, 40.6 g), EtOAc (55.0 g), and *n*-butanol (*n*-BuOH, 96.5 g) fractions, in addition to a H_2O residue (277.9 g).

Quantification of Total Phenol Contents. The total phenolic contents were determined via a modified version of Folin−Ciocalteu's method, using gallic acid as a standard (*21*). A 0.1 mL aliquot of the extract solution was mixed with 1 mL of Folin−Ciocalteu reagent [previously diluted with water 1:1 (v/v) and 2 mL of 20% sodium carbonate (Na_2CO_3) solution]. The mixed solution was maintained at room temperature for 45 min, followed by 10 min of centrifugation at 5000*g*. The absorbance of the supernatant was measured at 730 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). The total phenol contents of the fractions were expressed as a percentage (wt %) compared to the weight of the dried extract or fractions.

Isolation of Compounds. The EtOAc fraction (55.0 g) of *E. cava* was subjected to column chromatography over a silica gel with CH_2Cl_2/methanol (30:1 to 1:1), yielding 16 subfractions (EF01−EF16). The Sephadex LH-20 column chromatography of fraction 2 (EF02, 1.1 g) was conducted with methanol, thereby yielding 5 subfractions (EF0201−EF0205). Sephadex LH-20 column chromatography of fraction 1 (EF0201, 648.9 mg) using identical solvent conditions, was used to isolate phloroglucinol (600.0 mg). Dioxinodehydroeckol (88.0 mg) was purified from fraction 2 (EF0202, 110.4 mg), with Sephadex LH-20 (100% methanol). 7-Phloroeckol (43.4 mg) was isolated from fraction 11 (EF11, 135 mg) with RP-18 (20−100% methanol, gradient) and Sephadex LH-20 (100% methanol). The structural identities of phloroglucinol, dioxinodehydroeckol, and 7-phloroeckol were verified via a comparison to the published spectral data (*22*, *23*).

Enzyme Assay. The inhibitory effect on tyrosinase was measured using the spectrophotometric method developed by No et al. (*24*). A total of 10 μL of each sample solution with different concentrations and 20 μL of mushroom tyrosinase (1000 units/mL) in 50 mM phosphate buffer (pH 6.5) were added to 170 μL of an assay mixture containing a ratio of 10:10:9 of 1 mM L-tyrosine solution, 50 mM potassium phosphate buffer (pH 6.5), and distilled water in a 96-well microplate. The samples dissolved in dimethylsulfoxide (DMSO) were diluted 30-fold with distilled water prior to the experiment. After 30 min of incubation at 37 °C, the absorbance of the mixture was determined at 490 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). The extent of inhibition by addition of samples was expressed as the concentration required for a 50% inhibition (IC_{50}). The percentage inhibition of tyrosinase activity was calculated via the following equation:

$$\text{inhibition } \% = [\{1-(\text{Aa}-\text{Ab})/\text{Ac}\} \times 100]$$

in which Aa is the absorbance at 490 nm with the test sample and enzyme, Ab is the absorbance at 490 nm with the test sample and without enzyme, and Ac is the absorbance at 490 nm with enzyme and without the test sample. The pre-incubation mixture consisted of 60 μL of 50 mM phosphate buffer (pH 6.5), 50 μL of water, 10 μL of the sample solution, and 20 μL of the aqueous solution of the mushroom tyrosinase (1000 units/mL). The mixture was pre-incubated for 5 min at 37 °C. Then, 60 μL of 1 mM L-tyrosine was added, and the reaction was monitored at 490 nm after 2 min of incubation.

Kinetic Analysis. The reaction mixture consisted of five different concentrations of L-tyrosine (1−6 mM) as a substrate and mushroom tyrosinase in 50 mM potassium phosphate buffer. Each sample was added to the reaction mixture at several different concentrations, respectively. The Michaelis constant (K_m) and maximal velocity (V_{max}) of the tyrosinase were determined by Lineweaver−Burk plots using various concentrations of L-tyrosine as substrates.

Cell Culture. The murine melanoma B16F10 cells (CRL 6323) were acquired from the American type Culture Collection (ATCC, Manassas, VA). B16F10 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, 100 units/mL penicillin G, and 100 mg/mL streptomycin and cultured at 37 °C in a humidified atmosphere with 5% CO_2.

3-(4,5-Dimethyl-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. The cytotoxicity levels of compounds on melanoma B16F10 cells were assessed via the MTT method (*25*). The cells were grown in 96-well plates at a density of 1×10^5 cells/well. After 24 h, the cells were washed in fresh medium and treated with different concentrations of compounds. After 72 h of incubation, the cells were rewashed and 100 μL of MTT solution (5 mg/ml) was added and incubated for 4 h. Finally, DMSO (100 μL) was added to solubilize the formed formazan salt, and the amount of formazan salt was quantified by measuring the absorbance at 540 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the quantity of MTT converted into formazan salt. The viability of the cells was quantified as a percentage compared to the control (absorbance of treated cells − absorbance of blank/absorbance of control − absorbance of blank × 100), and dose−response curves were developed. The data were expressed as the means from at least three independent experiments.

Measurement of Melanin Contents. The melanin content was determined in accordance with the procedure described by Hosoi et al. (*26*), with some slight modifications. The B16F10 melanoma cells were seeded at a density of 2×10^4 cells per well in 24-well culture plates and then incubated for 24 h. The cells were treated with various concentrations of sample (12.5−100 μM). After 1 h, 100 μM 3-isobutyl-1-methylxanthine (IBMX) was added and incubated for 72 h. The cells were washed twice in phosphate-buffered saline and

dissolved in 1 N NaOH (in 10% DMSO) by 30 min of boiling (60 °C). The lysates were centrifuged for 5 min at 5000*g*, and then the absorbance value of the supernatant was measured at 405 nm.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD) values of three experiments. The means were statistically analyzed using Student's *t* test. Values of $p < 0.001$, 0.01, and 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In this study, we attempted to assess the tyrosinase inhibitory effect of the EtOH extract derived from *E. cava*, along with its solvent soluble fractions, including *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH, as well as a H_2O layer at concentrations of 50 μg/mL (data not shown). Among the partitioned fractions of the ethanolic extract, the EtOH extract (67.35 ± 0.28%) and the EtOAc-soluble fraction (79.97 ± 2.09%) evidenced noticeable tyrosinase inhibition activity. The EtOAc fraction exhibited more profound inhibitory activity than was noted with arbutin (57.13 ± 1.83%), which was employed as a positive control.

According to the results of a previous study, the EtOAc fraction of *Ecklona* sp. evidenced potent tyrosinase inhibitory properties. These results were attributed to a rich polyphenolic compound content, including phloroglucinol derivatives (*27*). We investigated the total phenol contents of the EtOH extract from *E. cava* and its solvent soluble fractions via Folin–Ciocalteu's method (**Table 1**). It can be clearly seen, in **Table 1**, that the phenolic contents of the EtOAc fraction (66.2%) were highest among the fractions.

Therefore, we conducted further phytochemical investigations to isolate bioactive compounds of the EtOAc fraction via repeated column chromatography over silica gel, Sephadex LH-20, and RP-18 gel, which led to the isolation of three compounds. The structure identification of these compounds was verified by 1D (^{1}H and ^{13}C NMR) and 2D (HMQC and HMBC) spectroscopic analyses and by comparisons to published data (*22*, *23*). The chemical structure of compounds **1**–**3** were identified as phloroglucinol (**1**, 0.103%), dioxinodehydroeckol (**2**, 0.015%), and 7-phloroeckol (**3**, 0.007%), respectively (**Figure 1** and **Table 2**).

The inhibitory activities of isolated compounds toward mushroom tyrosinase were also assessed (**Table 2**). Among the compounds, dioxinodehydroeckol (**2**) and 7-phloroeckol (**3**) exhibited inhibitory effects on tyrosinase-catalyzed L-tyrosine oxidation, with IC_{50} values of 222.94 and 0.85 μM. In particular, 7-phloroeckol (**3**) evidenced inhibitory effects 47- and 286-fold stronger than those of the positive controls, kojic acid (40.28 μM) and arbutin (243.16 μM), respectively. However, phloroglucinol (**1**) exerted no detectable effects on tyrosinase inhibition. The tyrosinase inhibitory effects of phloroglucinol (**1**) and dioxinodehydroeckol (**2**) have been previously reported (*27*). In the present study, it was demonstrated that these compounds also possess similar inhibitory effects toward tyrosinase as compared to the previous study. However, this is the first report, to the best of our knowledge, concerning the inhibitory effects of 7-phloroeckol (**3**) against mushroom tyrosinase.

A kinetic study of L-tyrosine oxidation catalyzed by mushroom tyrosinase was analyzed in the presence of 7-phloroeckol (**3**) by a Lineweaver–Burk plot (**Figure 2**). The K_i value of 7-phloroeckol (**3**) was estimated to be 2.5 μM at 3 μM and 2.7 μM at 5 μM. 7-Phloroeckol (**3**) evidenced the same K_m value of 1.3×10^{-3} M and V_{max} values of 5.3×10^{-3} and 4.1×10^{-3} ΔOD_{490}/min at 3 and 5 μM, respectively (**Table 3**). As the

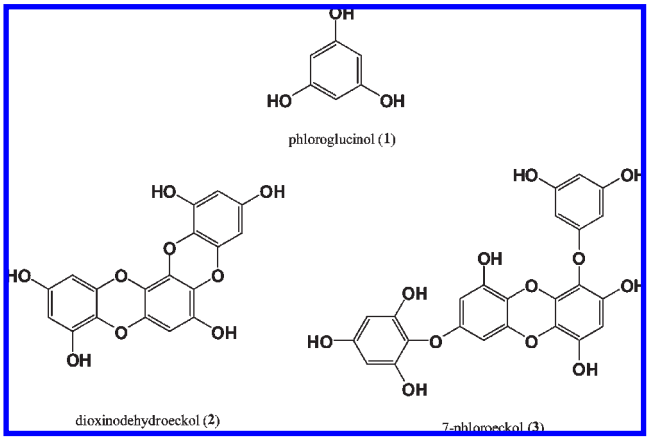

Figure 1. Structures of compounds isolated from *E. cava*.

Table 1. Total Phenol Contents of the Ethanolic Extract of *E. cava* and Its Solvent Soluble Fractions

samples	total phenol contents (%)[a]
EtOH extract	30.7
n-hexane fraction	10.8
CH_2Cl_2 fraction	15.6
EtOAc fraction	66.2
n-BuOH fraction	33.7
H_2O fraction	7.6

[a] Data were expressed as a percentage (wt %) compared to the weight of dried extract or fractions.

Table 2. Yields of Compounds Isolated from the EtOAc Fraction of *E. cava* Ethanolic Extract and Their Inhibition Effects on Mushroom Tyrosinase

samples	yields (%)[a]	IC_{50} (μM)[b]
phloroglucinol (**1**)	0.103	>300
dioxinodehydroeckol (**2**)	0.015	222.94
7-phloroeckol (**3**)	0.007	0.85
arbutin[c]		243.16
kojic acid[c]		40.28

[a] Yields were expressed as a percentage (wt %) of the compound/ethanolic extract (584.3 g). [b] Inhibitory effect was expressed as 50% inhibitory concentration obtained by interpolation of the concentration inhibition curve. [c] Arbutin and kojic acid were used as positive controls.

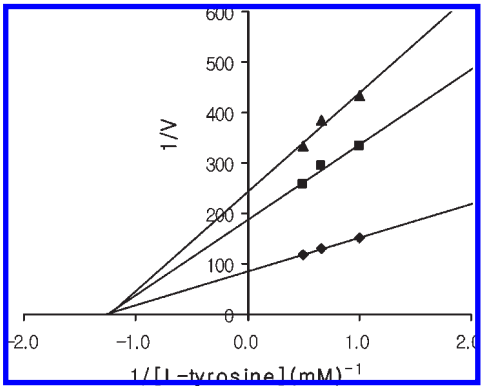

Figure 2. Lineweaver–Burk plot of mushroom tyrosinase in the presence of 7-phloroeckol. Data were expressed as the means of three independent tests with different concentrations of L-tyrosine used as a substrate. Concentrations of 7-phloroeckol as an inhibitor were as follows: ▲, 5 μM; ■, 3 μM; ◆, control.

substrate concentration varies, the V_{max} values of tyrosinase were reduced in a dose-dependent manner, without altering the binding affinity of the catalyst for the substrate. Therefore, 7-phloroeckol (**3**) was identified as a noncompetitive inhibitor of mushroom tyrosinase. In this mode of inhibition, the inhibitor caused a change in the structure and shape of the enzyme, and the modified enzyme was no longer capable of binding correctly with the substrate (*28*).

In this study, we also attempted to confirm whether phlorotannins can inhibit melanin biosynthesis in melanoma cells. We evaluated the effects of phlorotannins isolated from *E. cava* on melanin synthesis in B16F10 melanoma cells. The cell viability of the compounds was determined via a 3 day MTT assay. As is shown in **Figure 3**, the compounds exerted no cytotoxic effects on the melanoma cells in the concentration range of 12.5−100 μM.

First of all, we assessed the inhibitory effects of the compounds at 25 μM on IBMX-induced melanin production in B16F10 cells (**Figure 4**).

IBMX, a well-known stimulator of melanogenesis, is a potent cAMP phosphodiesterase inhibitor and increases cyclic adenosine monophosphate (cAMP) (*29*, *30*). cAMP is regarded as a key messenger in the regulation of melanin synthesis. It has been shown to induce significant elevations in melanin production following a single treatment in melanoma cells (*31*).

Among the compounds evaluated, only 7-phloroeckol (**3**) exhibited significant inhibitory effects on IBMX-mediated melanin synthesis. Therefore, we elected to further assess the inhibitory effects of 7-phloroeckol (**3**) on IBMX-induced melanin production in a concentration range of 12.5−100 μM. The melanin content of 7-phloroeckol-treated cells was reduced significantly in a dose-dependent manner, showing 90.6% at 12.5 μM, 67.8% at 25 μM, 57.2% at 50 μM, and 51.5% at 100 μM as compared to the IBMX-treated group (**Figure 5**). As a result, 7-phloroeckol was shown to function as a potent inhibitor on IBMX-induced melanin production in B16F10 melanoma cells.

In the present study, it was demonstrated that 7-phloroeckol exerted significant inhibitory effects on both mushroom tyrosinase and melanin synthesis in melanoma cells. 7-Phloroeckol had already been isolated from *Eisenia bicyclis* and *Ecklonia stolonifera*, and its bioactivities had been investigated, including antioxidant and antidiabetic complications and angiotensin-converting enzyme inhibitory effects (*19*, *24*).

Arbutin and kojic acid, which were used as positive controls, have been extensively used as cosmetic materials with skin-whitening effects and as medical agents for the treatment of a variety of cutaneous hyperpigmentation disorders (*2*). The depigmenting activities of these whitening agents have

Table 3. Kinetic Parameters of Mushroom Tyrosinase in the Presence of 7-Phloroeckol (**3**)

compound	K_m (M)	V_{max} (ΔOD_{490}/min)	K_i (μM)
7-phloroeckol (**3**)	1.3×10^{-3}		
3 μM		5.3×10^{-3}	2.5
5 μM		4.1×10^{-3}	2.7

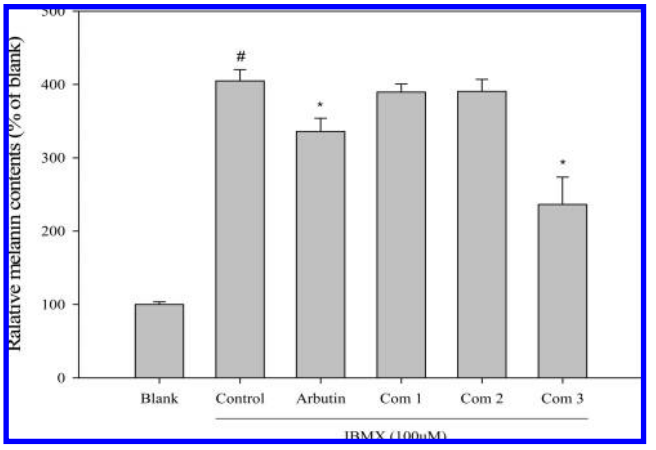

Figure 4. Inhibitory effects of compounds at 25 μM on IBMX-induced melanin production in B16F10 cells. The values are expressed as the means ± SD from three experiments. (#) $p < 0.001$ versus the blank group. (*) $p < 0.05$ versus the IBMX-treated group.

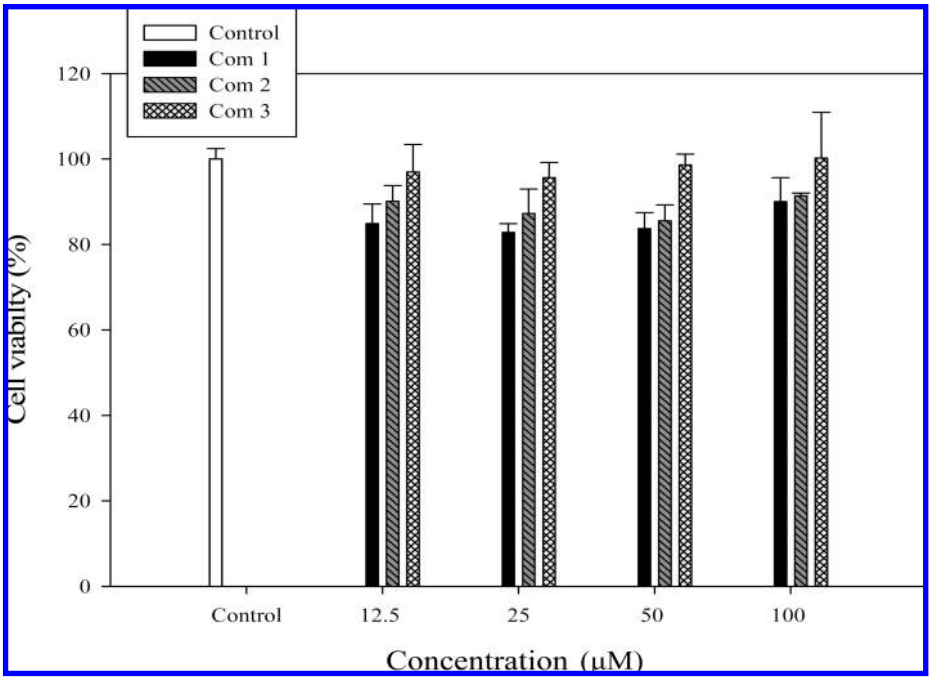

Figure 3. Effect of compounds on cell viability in B16F10 melanoma cells.

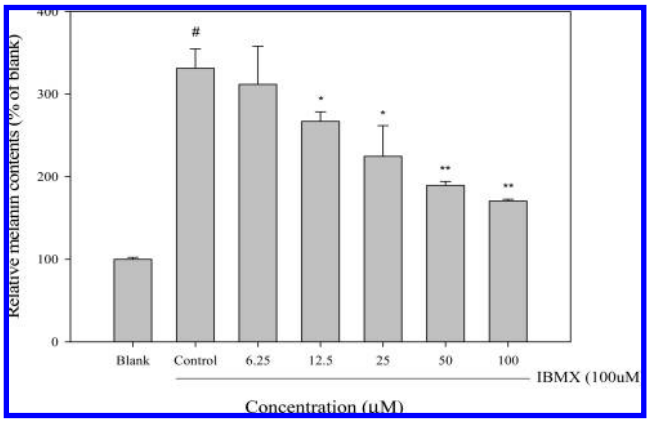

Figure 5. Inhibitory effects of 7-phloroeckol on IBMX-induced melanin production in B16F10 melanoma cells. The values are expressed as the means ± SD from three experiments. (#) $p < 0.001$ versus the blank group. (∗) $p < 0.05$ and (∗∗) $p < 0.01$ versus the IBMX-treated group.

been attributed to their ability to inhibit tyrosinase activity (*2*). However, according to the results reported in recent studies, arbutin exerted a low skin-whitening effect and kojic acid has serious side effects, including cytotoxicity, skin cancer, hepatocarcinogenesis, and dermatitis (*2*).

In conclusion, the results of the present study demonstrated that the ethanolic extract of *E. cava* and its component, 7-phloroeckol, exert potent inhibitory effects on mushroom tyrosinase. This compound also evidenced a significant inhibitory effect against IBMX-induced melanin synthesis in B16F10 melanoma cells. These results indicate that 7-phloroeckol from *E. cava* may be a good candidate as an inhibitor of melanin formation in cosmetic applications.

Furthermore, we plan to conduct additional studies into the mechanisms underlying the inhibitory properties of this compound.

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