Recovery of Low-molecular Weight Galloyltannins from Agricultural Residue of *Juglans sigillata* Dode Seed Husks and their Tyrosinase Inhibitory Effect

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The phytochemical investigation, including fractionation and purification of 70% acetone extracts of *Juglans sigillata* seed husks, an agricultural residue, led to the isolation of five low-molecular weight galloyltannins. The structures of the extractives were elucidated as 1,2,6-tri-*O*-galloyl-*β*-D-glucose (**1**), 3,4,6-tri-*O*-galloyl-*β*-D-glucose (**2**), 2,3,4,6-tetra-*O*-galloyl-*β*-D-glucose (**3**), 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**4**), and tannic acid (**5**), primarily based on their spectral (NMR and MS) and chemical evidence. Galloyltannins **1-5** showed strong inhibitory activity against mushroom tyrosinase, with IC_{50} values ranging from 35.27 to 76.37 μM; kojic acid, which was used as a positive control, had an IC_{50} value of 342.14 μM. It was further found that **1-5** inhibited melanin production and exhibited intracellular tyrosinase activity, as well as down-regulated mRNA and protein expression levels of tyrosinase, in B16F10 mouse melanoma cells. Therefore, the isolated extractives from seed husks of *J. sigillata* may serve as potential candidates for hyperpigmentation remediation and as skin-whitening agents in the cosmetics industry.

Keywords: Juglans sigillata; Extractives; Galloyltannin; Tyrosinase; B16F10 mouse melanoma cell

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INTRODUCTION

Tyrosinase is a member of the type three copper enzyme family, which is considered to be a key enzyme in melanin synthesis (Piao *et al.* 2002; Shiino *et al.* 2003). This enzyme catalyzes the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (DOPA), and further to dopaquinone, using the catalytic center (Baek *et al.* 2009). Melanin is essential for protecting human skin from ultraviolet radiation, but overproduction and accumulation of melanin in the skin induces pigmentation disorders (Pomerants 1963). Therefore, compounds with tyrosinase inhibitory activity have the potential to be applied in skin-lightening and depigmenting in the cosmetic field (Rout and Banerjee 2007; Zocca *et al.* 2010). Several anti-melanogenesis compounds, such as hydroquinone, azelaic acid, linoleic acid, kojic acid, and ellagic acid, which are isolated from natural resources, have been increasingly applied in cosmetics and pharmaceuticals

as a way to prevent overproduction of melanin in epidermal layers (Kai *et al.* 2009). Tyrosinase inhibitors from natural resources are generally considered to be free of harmful side effects and cost-effective; the isolation and identification of anti-tyrosinase compounds from natural sources continues to gain great interest (Kim *et al.* 2006; Lerch 1987).

Juglans sigillata Dode. (Juglandaceae), a fast growing deciduous tree, is indigenous in mountain regions of Tibet, Sichuan, Yunnan, and Guizhou provinces in southwest China (Wu *et al.* 1999). The seeds of *J. sigillata* are highly valued for their dietary nutrition, and their seed husks have long been used as folk medicines for the treatment of esophageal, gastric, cardiac, and lung cancer (Liu *et al.* 2010). The seed husk of *J. sigillata,* as an agricultural and food industry residue, is a rich resource for developing potential value-added medicines, healthcare agents, and cosmetics, due to its pharmaceutical properties (Ito *et al.* 2007). Previous investigation of *J. sigillata* fresh pericarps yielded 9 α-tetralone derivatives (Liu *et al.* 2010). However, to the best of our knowledge, the anti-tyrosinase activity of this species has never been reported. In order to validate the traditional use of *J. sigillata* and to clarify its pharmacological effects, the 70% acetone extract of the plant's seed husks were investigated with the aim of identifying the constituents responsible for the anti-tyrosinase effect. Solvent isolation and repeated column chromatography fractionation resulted in the purification and elucidation of five low-molecular weight galloyltannins from the extract's *n*-BuOH soluble fraction. This paper reports the isolation of these extractives from *J. sigillata*, as well as their tyrosinase inhibitory effects.

EXPERIMENTAL

Plant Material

The seed husks of *J. sigillata* were collected in Santai County, Yunnan province, China, in August 2009, and were authenticated by Dr. Dan Wang (Institute of Chemical Industry of Forest Products, China). A voucher specimen (No. 200908002) was deposited at the herbarium of Tianjin Key Laboratory of Pulp & Paper, Tianjin University of Science & Technology, China.

Instruments

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in MeOH-d_4 with TMS as an internal standard using a Bruker Avance DPX 400 spectrometer. EI and positive FAB-MS spectroscopy was done with a Micromass Autospec M363 spectrometer, and MALDI-TOF-MS spectroscopy was measured on a Model Voyager-DE STR spectrometer.

Thin layer chromatography (TLC) analysis were carried out on DC-Plastikfolien Cellulose F (Merck Co.) plates and developed with *t*-BuOH-HOAc-H_2O (3:1:1, v/v/v, solvent A) and HOAc-H_2O (3:47, v/v, solvent B). The TLC spots were detected by UV light (254 and 365 nm) and by spraying with a 1% $FeCl_3$ (in EtOH) solution, followed by heating.

Extraction and Fractionation

The air-dried and finely ground seed husks (3.08 kg) of *J. sigillata* were extracted with 70% acetone at room temperature more than three times (each 20 L for 72 h) to

obtain a sufficient amount of extracts. As demonstrated in Fig. 1, the resulting extracts were combined, filtered, and concentrated with a rotary evaporator *in vacuo* to give an aqueous residue, which was then sequentially fractionated with a series of liquids with polarity gradients and finally freeze-dried to give *n*-hexane- (4.81 g, yield 0.16%), CH_2Cl_2- (3.31 g, yield 0.11%), EtOAc- (36.15 g, yield 1.17%), *n*-BuOH- (42.82 g, yield 1.39%), and H_2O- (149.30 g, yield 4.85%) soluble fraction powders. The powders were kept at -4 °C, in darkness, and in sealed vials for further experiments.

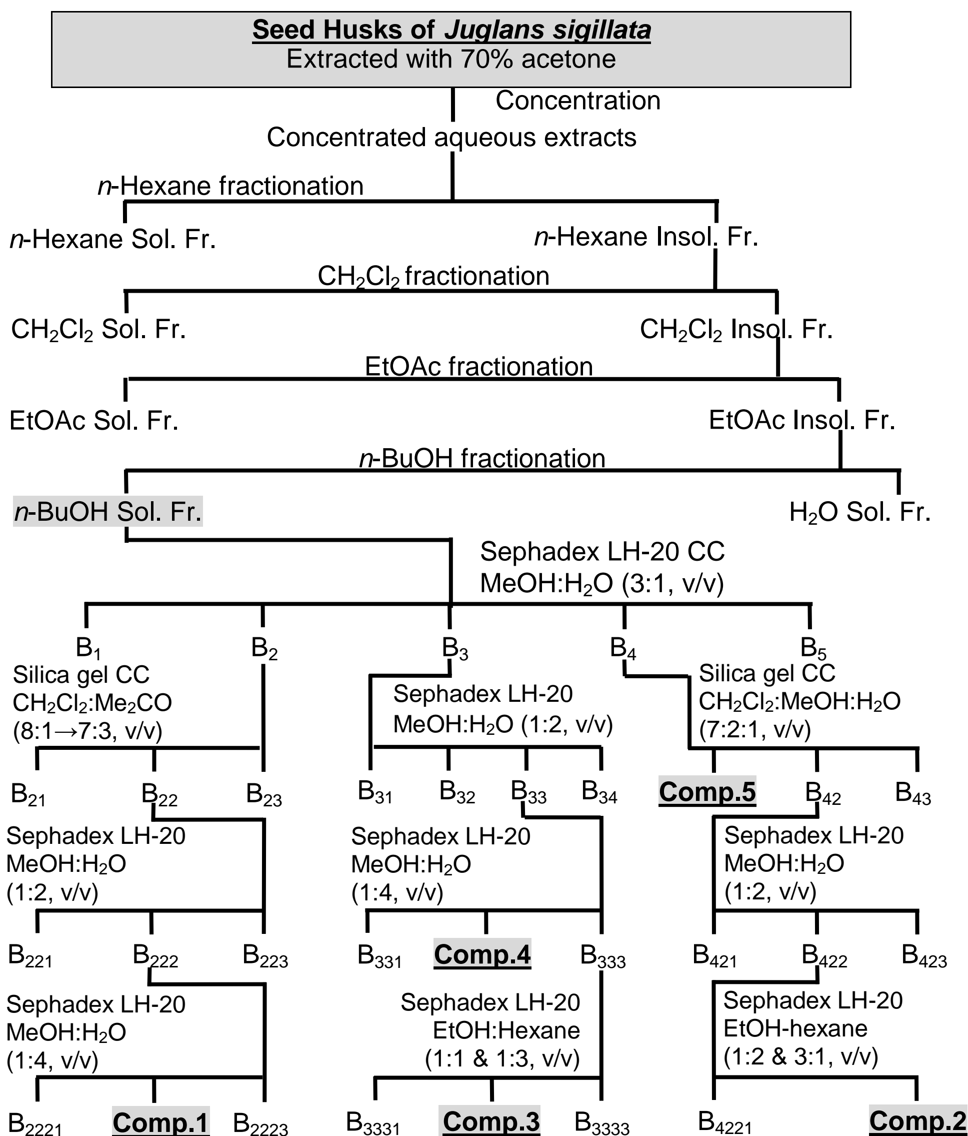

Fig. 1. Extraction, fractionation, and purification of galloyltannins from seed husks of *J. sigillata*

Isolation of Low-molecular Weight Galloyltannins

As shown in Fig. 1, a portion of the resulting *n*-BuOH soluble fraction (32.81 g) was applied to a Sephadex LH-20 open column chromatography (CC) eluting with MeOH-H_2O (3:1, v/v) to give five main fractions: B_1 (3.29 g), B_2 (10.11 g), B_3 (4.12 g), B_4 (13.88 g), and B_5 (1.20 g), which were grouped and monitored by TLC performance. Fraction B_2 was loaded to a silica gel open column using a gradient of CH_2Cl_2-acetone (8:1→7:3, v/v) as the solvent system for further purification to yield three subfractions.

The second subfraction (B_{22}) was stepwisely rechromatographed with MeOH-H_2O (1:2 and 1:4, v/v) on a Sephadex LH-20 open column to yield 81 mg of yellow amorphous compound **1**. Fraction B_4 was subjected to a silica gel open column and eluted with CH_2Cl_2-MeOH-H_2O (7:2:1, v/v) for further separation to give compound **5** (79 mg), B_{42} (10.25 g), and B_{43} (1.62 g). B_{42} was successively subjected to a Sephadex LH-20 open column eluted with MeOH-H_2O (1:2, v/v) and EtOH-hexane (1:2 and 3:1, v/v) to give yellow amorphous compound **2** (52 mg). Fraction B_3 was subjected to repeated Sephadex LH-20 open column chromatography using MeOH-H_2O (1:2 and 1:4, v/v) and EtOH-hexane (1:1 and 1:3, v/v) as eluting solvents to obtain 48 mg of compound **3** and 63 mg of compound **4** as yellow amorphous compounds.

Mushroom Tyrosinase Assay

The effects of the extractives on mushroom tyrosinase activity were investigated by a spectrophotometric method described in the literature (Zhang *et al*. 2009). Briefly, a total volume of 200 μL of assay mixture containing 80 μL of phosphate buffer (pH 6.8), 40 μL of mushroom tyrosinase (500 U/mL), and 40 μL of test compounds was added to a 96-well plate and incubated at 25 °C for 5 min. Subsequently, a substrate solution (25 mM L-DOPA, 40 μL) was added. After incubation for 10 min at 25 °C, the amount of dopachrome formed in the reaction mixture was determined by measuring the absorbance at 490 nm in a microplate reader (SpectraMax 250). Kojic acid was used as a positive standard. Data were recorded as a percentage of inhibition using the following formula:

$$\text{Inhibition (\%)} = [(A_{test\ sample} - A_{blank}) / A_{control}) \times 100]\ \%$$

where $A_{control}$ is the OD at 490 nm with enzymes, but without the test sample; A_{blank} is the OD at 490 nm with the test sample, but without enzymes; and $A_{test\ sample}$ is the OD at 490 nm with both the test sample and enzymes.

Cell Line and Cell Culture

B16F10 mouse melanoma cells obtained from American Type Culture Collection were grown in dulbecco's modified eagle medium (DMEM) supplemented with 10% phosphate buffer solution (PBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Cell Viability Assay

The cytotoxicity of extractives on B16F10 mouse melanoma cells was investigated. Cells were seeded into 96-well plates at a density of 1×10^5 cells/well for 16 h and then exposed to medium in the presence of 50 μM of each compound for 24 h. After removing the supernatant of each well, a total of 10 μL of MTT solution (5 mg/mL in PBS) and 90 μL of FBS-free medium were added to each well at the time of incubation for 4 h at 37 °C. The dark blue formazan crystals that formed inside the intact mitochondria were solubilized with 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stop solution (containing 10% sodium dodecyl sulfate (SDS)) and 0.01 M hydrochloric acid. The amount of MTT formazan was quantified by a microplate reader at 550 nm. The optical density of formazan formed in control cells was taken as 100% viability. Cell viability was expressed as a percentage of the control culture value.

Determination of Melanin Content in B16F10 Mouse Melanoma Cells

B16F10 mouse melanoma cells (2×10^5 cells) were grown in a 6-well plate for measurement of intracellular melanin content. After 32 h of incubation, cells were treated with 50 μM of each compound for 24 h and then harvested and washed twice with cold PBS. Finally, the cell pellet was dissolved in 0.5 mL of 1 N NaOH at 90 °C for 1 h. The lysates were centrifuged for 10 min at 3,000 g. The protein content in the supernatant was determined by Bradford assay (Bradford 1976). The relative melanin content was measured at 405 nm in a microplate reader. Results from samples were analyzed as a percentage of the control.

Assay of Cellular Tyrosinase Activity

Tyrosinase activity in B16F10 mouse melanoma cells was determined by measuring the rate of oxidation of L-DOPA. B16F10 mouse melanoma cells (4×10^5 cells) were seeded in a 6-well plate. After 32 h of incubation, cells were treated with 50 μM of each compound for 24 h and then harvested and washed twice with cold PBS. Finally, the cell pellet was lysed in 0.2 mL of PBS buffer (pH 6.8) containing 1% (w/v) Triton X-100 and 1 mM phenylmethanesulfonylfluoride (PMSF). One hundred μL of each cell extract was placed in a 96-well plate, and the enzymatic assay was commenced by adding 100 μL of L-DOPA (2 mM); dopachrome formation in the reaction mixture was monitored at 490 nm in a microplate reader after 60 min at 37 °C. Results from samples were analyzed as a percentage of the control.

RNA Preparation, Semi-quantitative Reverse Transcriptase- and Real-time Polymerase Chain Reactions (RT-PCR)

B16F10 mouse melanoma cells (2×10^6 cells) were incubated in a 6-well plate. After 16 h of incubation, cells were treated with 50 μM of each compound and then harvested and washed twice with cold PBS. Total RNA was isolated with Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA (2 μg) was incubated with oligo-dT15 and dNTPs for 5 min at 65 °C and then mixed with a 5× first-strand buffer and 0.1 M dithiothreitol (DTT). The reaction mixture was further incubated for 5 min at 37 °C and for 60 min after the addition of murine leukemia virus reverse transcriptase. Reactions were terminated after 10 min at 70 °C. The primers used in this experiment were as follows: GAPDH sense 5'-CAC TCA CGG CAA ATT CAA CGG CAC-3', antisense 5'-GAC TCC ACG ACA TAC TCA GCA C-3', Tyrosinase sense 5'-GGC CAG CTT TCA GGC AGA GGT-3', and antisense 5'-TGG TGC TTC ATG GGC AAA ATC -3'. Quantification of mRNA was also performed using a real-time RT-PCR following the manufacturer's instructions with SYBR Premix Ex Taq using a real-time thermal cycler.

Western Blot Analysis

B16F10 mouse melanoma cells (2×10^6 cells) were incubated in a 6-well plate. After 32 h of incubation, cells were treated with 50 μM of each compound for 24 h and were harvested and washed twice with cold PBS. After centrifugation, cells were lysed in 200 μL of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM ethylene diamine tetraacetic acid (EDTA), 2 mM ethyleneglycotetraacetic acid, 50 mM *β*-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1% Triton X-100, 10% glycerol, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 1 mM benzimide, and 2 mM hydrogen peroxide). The lysate was incubated on ice for 10 min, followed by sonication for 5 s. The lysate was then

centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was collected, and the protein concentration was determined using the Bradford assay. Aliquots of the lysates (20 µg of protein) were heated at 95 °C for 5 min, separated on 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF; Bio-Rad) membranes. The membranes were blocked in blocking buffer (Tris-buffered saline containing 3% BSA, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20) for 60 min at room temperature. The membrane was incubated for 60 min with the appropriate primary antibody at room temperature and then washed three times with the TBST buffer (Tris-buffered saline containing 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20), incubated further for 60 min with HRP-conjugated secondary antibodies, and washed three times with TBST buffer. Bound antibodies were visualized using an ECL detection kit (Amersham). Bands were visualized using a Mini BIS image analysis system and quantified with Gel Quant software.

Statistical Analyses

All tests were carried out in triplicate (n = 3). The data are expressed as the mean ± standard derivation (SD). One-way analysis of variance (ANOVA) was used to determine the significant differences between the groups, followed by a Dunnett's t-test for multiple comparisons. Values of *$p < 0.05$ were considered significant. All analyses were performed using SPSS for Windows 7, version 19.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Identification of Low-molecular Weight Galloyltannins

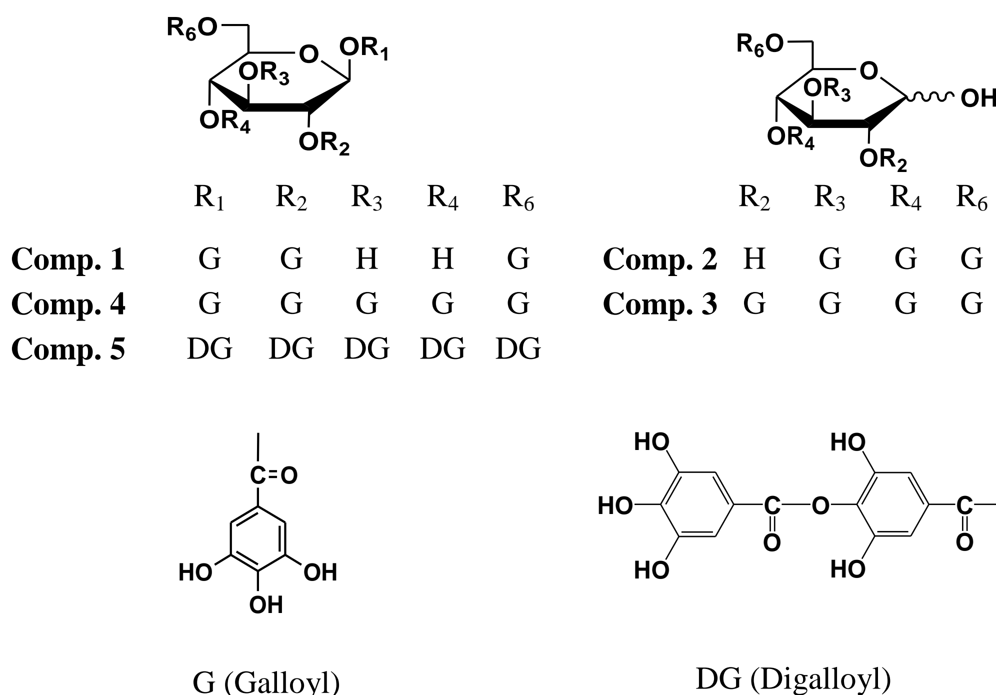

Fig. 2. Structures of the identified low-molecular weight galloyltannins from *J. sigillata* seed husks: 1,2,6-tri-*O*-galloyl-*β*-D-glucose (**1**), 3,4,6-tri-*O*-galloyl-*β*-D-glucose (**2**), 2,3,4,6-tetra-*O*-galloyl-*β*-D-glucose (**3**), 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**4**), and tannic acid (**5**)

Five low-molecular weight extractives were isolated from *J. sigillata* seed husks and their structures, as shown in Fig. 2, and were elucidated as 1,2,6-tri-*O*-galloyl-*β*-D-glucose (**1**) (Nonaka *et al.* 1981), 3,4,6-tri-*O*-galloyl-*β*-D-glucose (**2**) (Haddock *et al.* 1982), 2,3,4,6-tetra-*O*-galloyl-*β*-D-glucose (**3**) (Haddock *et al.* 1982), 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**4**) (Duan *et al.* 2004), and tannic acid (**5**) (Qiao and Li 2011), based on their chemical and spectroscopic data. In addition, compounds **1-5** are all galloyltannins. It is noteworthy that compounds **2**, **3**, and **5** were isolated as anomeric mixtures.

Mushroom Tyrosinase Inhibition Activity of the Compounds

The tyrosinase inhibitory activity of galloyltannins **1-5**, along with kojic acid, was examined using mushroom tyrosinase with L-DOPA as a substrate. Each compound assayed was prepared at varying concentrations, and their relative activities were expressed as IC_{50} values. Results presented in Table 1 demonstrate that **1-5** showed strong inhibitory activity against mushroom tyrosinase, with IC_{50} values of 74.76, 76.37, 57.89, 47.63, and 35.27 μM, respectively, whereas the IC_{50} value of kojic acid (a well-known tyrosinase inhibitor) was 342.14 μM. To the best of our knowledge, this is the first report of compounds **1-4** displaying significant inhibitory activity against mushroom tyrosinase.

Table 1. Mushroom Tyrosinase Inhibitory Activities of the Galloyltannins from Seed Husks of *J. sigillata* and Kojic Acid

Compound ID	IC_{50} value (μM)
1	74.76±5.28[d]
2	76.37±3.24[d]
3	57.89±4.26[c]
4	47.63±8.52[b]
5	35.27±3.78[a]
kojic acid [a]	342.14±23.43[e]

[a] Positive control; each value is expressed as the mean ± SD (*n* = 3)

The tyrosinase inhibitory results revealed that tannic acid (**5**), which bears 10 galloyl groups, exhibited the strongest effects (IC_{50} = 35.27±3.78 μM), followed by 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**4**) (IC_{50} = 47.63±8.52 μM), with 5 galloyl moieties, 2,3,4,6-tetra-*O*-galloyl-*β*-D-glucose (**3**) (IC_{50} = 57.89±4.26 μM), with 4 galloyl groups, and tannins with 3 galloyl groups, including 3,4,6-tri-*O*-galloyl-*β*-D-glucose (**2**) (IC_{50} = 76.37±3.24 μM) and 1,2,6-tri-*O*-galloyl-*β*-D-glucose (**1**) (IC_{50} = 74.76±5.28 μM). Related to structure-activity relationships (SARs), increasing numbers of galloyl groups in these tannins enhanced the inhibitory effect. To gain more chemical insight of the structure-activity relationship, more derivatives should be assessed; nevertheless, our study indicated that galloyl groups may play a critical role in the inhibitory activity of galloyltannins against tyrosinase.

Effect of Galloyltannins 1–5 on Cell Viability, Melanin Synthesis, and Tyrosinase Activity in B16F10 Mouse Melanoma Cells

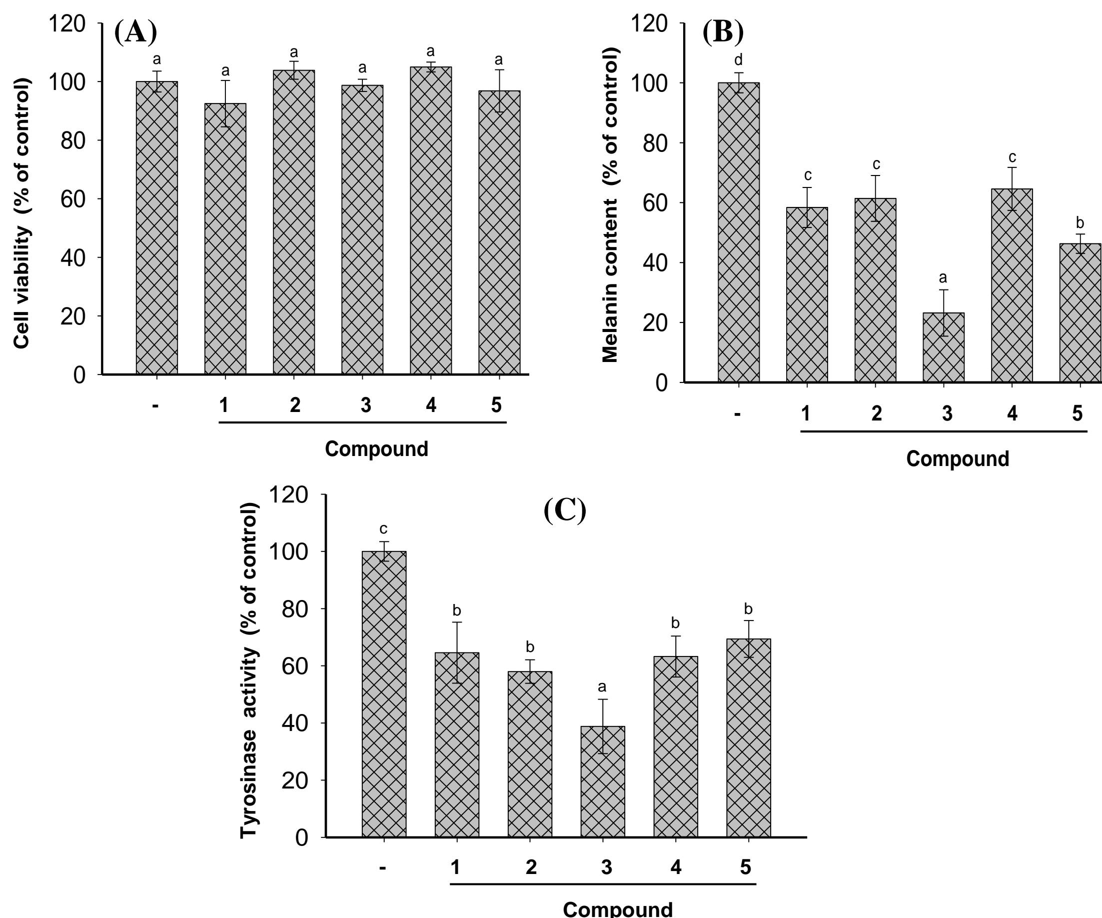

Fig. 3. Effect of galloyltannins **1**–**5** on cell viability, melanogenesis, and tyrosinase activity in B16F10 mouse melanoma cells. The cell viability (A), melanin content (B), and tyrosinase activity (C) were determined. Each value is expressed as the mean ± SD (n = 3). Values marked "a", "b", and "c" mean significantly different ($p < 0.05$)

As shown in Fig. 3 B and C, galloyltannins **1**–**5** exhibited varying degrees of inhibition on melanin content and intracellular tyrosinase activity in B16F10 mouse melanoma cells, and the inhibition percentage was higher than that of arbutin, a material in commercial whitening cosmetics (data not shown). These findings demonstrate that galloyltannins **1**–**5** regulated tyrosinase and subsequently suppressed melanin synthesis in B16F10 mouse melanoma cells at nontoxic doses. Amongst these compounds, compound **5** was found to possess highest tyrosinase inhibitory activity, whereas compound **3** exhibited the highest inhibition activity on melanin content and intracellular tyrosinase. There are several reasons to account for these differences: (i) mushroom tyrosinase is a cytosolic enzyme, while mouse tyrosinase is membrane-bound; (ii) compounds that inhibit cellular tyrosinase must first penetrate the melanocyte cell wall (Han *et al.* 2011); and (iii) melanogenesis can be controlled during inhibition of tyrosinase gene expression and direct inactivation of the enzyme.

Effect of Galloyltannins 1–5 on Tyrosinase Expression Level in B16F10 Mouse

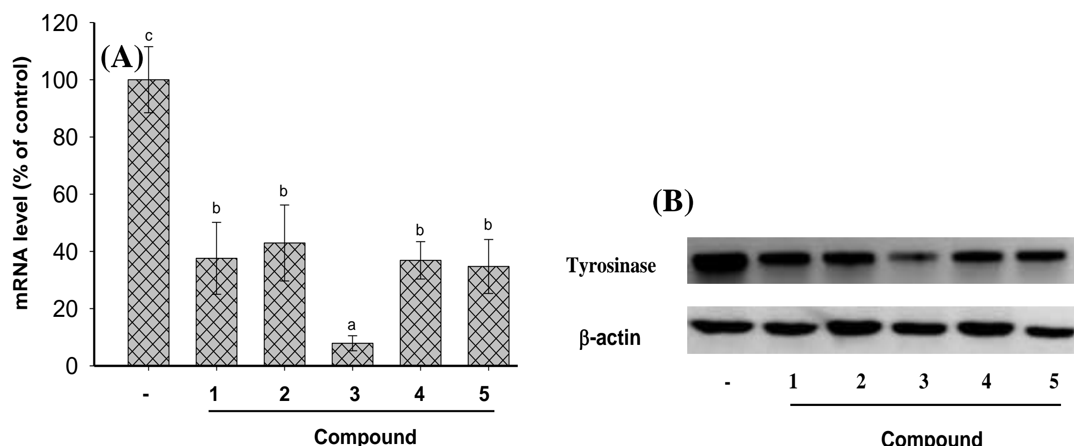

Fig. 4. Effect of compounds **1**–**5** on mRNA and protein levels of tyrosinase in B16F10 mouse melanoma cells. Cells (2×10^6) were treated with 100 μM of each compound for 24 h. Levels of mRNA and protein for tyrosinase were determined by real time-PCR (A) and western blot (B). These experiments were performed in triplicate, and the averaged results are presented

To determine the mechanism underlying the anti-melanogenic effects of galloyltannins **1**–**5**, B16F10 mouse melanoma cells were tested for the expression of the tyrosinase. As shown in Fig. 4, real-time PCR and western blot analysis showed that galloyltannins **1**–**5** markedly down-regulated mRNA and the protein expression levels of tyrosinase. Melanogenesis can be regulated at many steps: (1) inhibition of tyrosinase expression on the transcriptional or translation level together with inhibition of the melanogenic pathways, and (2) direct inhibitory effect on the enzyme. Thus, the anti-melanogenesis mechanism of compounds **1**–**5** was not only related to their inhibitory effect on the signaling pathway by regulating the transcriptional and translational expression of the tyrosinase gene, but also their inhibitory effect on enzyme. These results also indicate that galloyltannins **1**–**5** regulate tyrosinase and subsequently inhibit melanin synthesis in melanocytes.

CONCLUSIONS

1. In this study, the phytochemical composition of the *n*-BuOH-soluble fraction of *J. sigillata* seed husks was systematically investigated, and five extractives were isolated and structurally elucidated.
2. Through *in vitro* mushroom tyrosinase inhibition screening, we found that galloyltannins **1-5** exhibited tyrosinase inhibitory activity with low IC_{50} values. They also showed potent depigmenting ability against B16F10 melanoma cells, which indicated that the constituents might be promising candidates as skin-whitening agents in cosmetic formulations.
3. Furthermore, this work opens the door for more opportunities for further exploitation of the agricultural and food residues of *J. sigillata*.

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