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Whitening Activity of Phenolic Compounds from Rhizome of *Phragmites communis*

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Abstract – Activity guided phytochemical examination of the rhizome of *Phragmites* communis has led to the isolation of two phenolic acids and two lignans. Structures of these compounds were elucidated as methyl gallate (**1**), ρ-hydroxy cinnamic acid (**2**), (+) -lyoniresinol (**3**), (+)-lyoniresinol-9’-O-*β*-D-glucopyranoside (**4**) by comparisons with previously reported spectral data. To investigate the melanogenesis inhibitory effects of these compounds, the melanin level and tyrosinase activity were examined in B16F10 melanoma cell. Each compound inhibited both tyrosinase activity and melanin synthesis compared with positive control, kojic acid and arbutin. These results suggest that the phenolic compounds from *Phragmites communis* might be developed as a potent skin whitenting cosmeceuticals.
Keywords – *Phragmites communis*, Phenolic acid, Lignan, Melanogenesis inhibition, Tyrosinase activity, B16F10 melanoma cell.

Introduction

Phragmitis Rhizoma, rhizome of reed, grows on the swampy land and have been used for oriental traditional medicine to remedy for esophageal cancer, vomiting, lung abscess, tetrodotoxin poisoning, fever and diuresis.[1] From the Phragmitis Rhizoma, β-sitosterol and ρ-cinnamic acid were isolated and anti-hyperlipidemic effects were reported in recent study.[2]

Tyrosinase, known as polyphenol oxidase (PPO), is a multifunctional copper-containing enzyme widely distributed in plants and animals. Tyrosinase is also known to be a key enzyme for melanin biosynthesis and is responsible for melanization in animals and browning in plants.[3] Tyrosinase inhibitors may therefore be clinically useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation and also important in cosmetics for whitening and depigmentation after sunburn.[4]

As continuous studies on whitening agents from natural sources,[5,6] activity guided isolation on Phragmitis Rhizoma was preformed and investigated the inhibitory activities on tyrosinase and melanin biosynthesis in B16F10 melanoma cell.

Experimental

General experimental procedures – ^{1}H-(500 MHz) and ^{13}C-(125 MHz) NMR spectra were obtained on a Varian Unity INOVA 500 spectrometer (Varian, Inc., U.S.A.). Chemical shifts were expressed in parts per million (ppm) relative to TMS as an internal standard, and coupling constants (*J*) were given in Hz. MS were obtained on a Varian Saturn 4D mass spectrometer (Varian, Inc., U.S.A.) and JEOL JMS HX-110/110A tandem mass spectrometer (JEOL Ltd., Japan). TLC was carried out on Merck silica gel F_{254}-precoated glass plates and RP-18 F_{254s} plates.

Reagents – Tyrosinase (mushroom), L-3,4-dihydroxy-L-phenylalanine (L-DOPA), α-melanocyte stimulating hormone (α-MSH), melanin (synthetic), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], DMSO were obtained from Sigma (St. Louis, MO, USA). Arbutin was

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purchased from Merck (Germany).

Plant material – Phragmitis Rhizoma was collected from WanSan-Dong, YungChun-City, Kyung Buk, South Korea in June of 2007. A voucher specimen (2007614) has been deposited at the herbarium, College of Pharmacy, Chung-Ang University.

Extraction and isolation – Phragmitis Rhizoma (5 kg) were extracted with 80% aqueous acetone (3 × 10 L) for 3 days. After removal of Me_2CO in vacuo, the aqueous solution was filtered. The filtrate was concentrated and then applied to a column of Sephadex LH-20 (450 g, 10 × 70 cm). Elution with H_2O containing increasing proportion of MeOH afforded 5 fractions.

Repeated column chromatography of fraction 3 which showed potent inhibitory activity on tyrosinase over MCI gel with a H_2O/50% MeOH gradient, followed by YMC-ODS column chromatography with H_2O/50% MeOH gradient yielded **1** (20.6 mg). After the residue on YMC ODS gel (400/230 mesh) low pressure liquid column chromatography (H_2O/50% MeOH, gradient system) and Sephadex LH-20 column chromatography (H_2O/50% MeOH, gradient system) yielded **3** (4.3 mg) and **4** (22.4 mg). Column chromatography of fraction 4 which also showed potent activity on tyrosinase inhibition over MIC gel from 20% MeOH to 80% MeOH gradient, YMC ODS gel with H_2O/50% MeOH and following by recrystallization yielded **2** (102 mg).

Methyl gallate (**1**) – brown amorphous powder, Negative LC MS: *m/z* 183 $[M-H]^-$, ^{1}H-NMR (300 MHz, MeOH-d_4): δ 3.81 (3H, s, -OCH_3), 7.05 (2H, s, H-2, 6). ^{13}C-NMR (75 MHz, MeOH-d_4): δ 169.3 (C-7), 146.7 (C-3, 5), 139.9 (C-4), 121.6 (C-1), 110.1 (C-2, 6), 52.3 (-OCH_3)

***p*-Hydroxy cinnamic acid** (**2**) – brown amorphous powder, Negative LC MS : *m/z* 163 $[M-H]^-$, ^{1}H-NMR (300 MHz, MeOH-d_4): δ 6.28 (1H, d, J = 15.9 Hz, H-8), 6.80 (2H, d, J = 8.7 Hz, H-3,5), 7.59 (2H, d, J = 8.7 Hz, H-2,6), 7.60 (1H, d, J = 15.9 Hz, H-7), ^{13}C-NMR (75 MHz, MeOH-d_4): δ 171.3(C-9), 161.4 (C-4), 146.9 (C-7), 131.3 (C-2, 6), 127.4 (C-1), 117.0 (C-3, 5), 115.8 (C-8)

(+)-Lyoniresinol (3) – brown amorphous powder, Negative LC MS: *m/z* 417 $[M-H]^-$, ^{1}H-NMR (600 MHz, Me_2CO-d_6 + D_2O): δ 1.59 (1H, m, H-8), δ 1.95 (1H, m, H-8'), δ 2.55 (1H, dd, J = 12.6, 14.5 Hz, H-7ax), δ 2.65 (1H, dd, J = 4.49, 14.5 Hz, H-7eq), 3.33 (3H, s, 4-OCH_3), 3.49 (2H, d, J = 6.0 H-9'), 3.52 (1H, m, H-9a), 3.59 (1H, m, H-9b), 3.69 (6H, s, 3', 5'-OCH_3), 3.80 (3H, s, 5-OCH_3), 4.26 (1H, d, J = 6.0 Hz, H-7'), 6.40 (2H, s, H-2',6'), 6.55 (1H, s, H-6), ^{13}C-NMR (150 MHz, Me_2CO-d_6 + D_2O): δ 146.9 (C-5), 147.5 (C-5', 3'), 146.2 (C-3), 138.3 (C-1'), 137.7 (C-4), 133.8 (C-4'), 129.0 (C-1), 125.5 (C-2), 106.4 (C-6), 106.3 (C-2', 6'), 65.5 (C-9), 62.8 (C-9'), 58.8 (4-OCH_3), 55.9 (3', 5'-OCH_3), 55.5 (5-OCH_3), 48.2 (C-8'), 41.5 (C-7'), 38.8 (C-8), 32.8 (C-7)

(+)–Lyoniresinol-9'-*O*-*β*-D-glucopyranoside (**4**) – brown amorphous powder, Negative LC MS: *m/z* 581 $[M-H]^-$, ^{1}H-NMR (600 MHz, Me_2CO-d_6 + D_2O): δ 1.65 (1H, m, H-8), 2.07 (1H, m, H-8'), 2.55 (1H, dd, J = 4.5, 14.5 Hz H-7ax), 2.64 (1H in total, dd, J = 4.2, 14.5 Hz H-7eq), 3.25 (1H, m, glc-2), 3.27 (3H, s, 4-OCH_3), 3.31 (1H, s, glc-4), 3.29 (2H in total, m, H-9), 3.43 (1H , s, glc-3) 3.62 (2H in total, m, H-9), 3.79-3.59 (1H, m, glc-6), 3.69 (6H in total, s, 3',5'-OCH_3), 3.78 (3H, s, 5-OCH_3), 4.31 (1H, d, J = 7.5, glc-1), 4.34 (1H, m, H-7'), 6.42 (2H, s, H-2',6'), 6.54 (1H, s, H-6), ^{13}C-NMR (150 MHz, Me_2CO-d_6 + D_2O): δ 148.2 (C-3',5'), 147.7 (C-5), 146.8 (C-3), 138.9 (C-1'), 138.3 (C-4), 134.2 (C-4'), δ 129.7 (C-1), 126.1 (C-2), 107.3 (C-6), 106.7 (C-2',6'), 104.1 (glc-1), 77.4 (glc-3), 77.2 (glc-5), 74.4 (glc-2), 71.0 (glc-4), 70.8 (C-9'), 65.4 (C-9), 62.2 (glc-6), 59.6 (3-OCH_3), 56.7 (3',5'-OCH_3), 56.3 (5-OCH_3), 46.1 (C-8'), 42.3 (C-7'), 40.0 (C-8), 33.5 (C-7)

Tyrosinase-inhibition – Tyrosinase-inhibition assays were performed according to a modification of the method developed by Hearing.[7] Phosphate buffer (0.067 M) was prepared by adding 1.19 g of $Na_2HPO_4 \cdot 12H_2O$ to 50 ml of distilled water and the pH was adjusted to 6.8 using phosphoric acid. Samples of L-DOPA (25 μl and 50 μl) were added to a 50-μl aliquot of phosphate buffer. After mixing well, 25 μl of tyrosinase (100 units/ml) was added and the mixture was incubated for 10 min at 25 °C. The enzyme reaction was terminated by immersing the mixture in an ice bath. The absorbance of the resultant DOPA chromophore was measured at 492 nm.

Cell culture – Mouse B16F10 melanoma cells were purchased from the Korean Cell Line Bank. The cells were grown at 37 °C in a humidified atmosphere (5% CO_2) in a DMEM medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum, 100 IU/ml penicillin G and 100 mg/ml streptomycin (Gibco BRL, Grand Island, NY, USA).[8] And mouse B16F10 melanoma cells were used after cell counting with hemocytometer.

MTT assay – The cytotoxicity was measured by the mitochondrial-dependent reduction of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide] to formazan.[8] The cells were seeded at a density 1×10^4 cells/ml in 96 well-plates. After incubating for 2 h, the cells were treated with the samples (12.5, 25. 50 and 100 μg/ml). The cells were incubated for an additional 24 h, and the medium was replaced with fresh medium. The medium contained MTT (final concentration: 0.5 mg/ml), and the incubation continued for a further 1 h at 37 °C.

The medium was then removed and the MTT-formazan produced was dissolved in 200 µl DMSO. The extent of the reduction of MTT to formazan within the cells was quantified by measuring the absorbance at 540 nm using an ELISA reader (TECAN, Salzburg, Austria).

Inhibitory activity on tyrosinase in mouse B16F10 melanoma cells – Mouse B16F10 melanoma cells were cultured in a 96-well plate and incubated for 2 h at 37 ºC in a humidified atmosphere (5% CO_2). The cells were then incubated in a medium containing samples. After incubating for an additional 48 h, the cells of each well was washed with 10 mM PBS and was dissolved with 1% Trition X-100 (Sigma, St. Louis, MO, USA) in 10 mM PBS. After centrifuging on 1000 rpm, the supernatants were used as enzymatic solution. 2 mg/ml L-DOPA was added to each enzymatic solution during 1 hr in a 96-well plate, and the extent of the oxidation of L-DOPA to DOPAchrome was quantified by measuring the absorbance at 492 nm using an ELISA reader. Tyrosinase activity on mouse B16F10 melanoma cell was calculated as sample *O.D.* / control *O.D.* × 100.[9]

Inhibitory activity on melanin production in mouse B16F10 melanoma cells – Mouse B16F10 melanoma cells were cultured in a 96-well plate and incubated for 2 h at 37 ºC in a humidified atmosphere (5% CO_2). The cells were then incubated in a medium containing samples. After incubating for an additional 48 h, the cells of each well were washed with 10 mM PBS and was dissolved with 0.2 M NaOH for 1 hr at 60 ºC. The melanin production was quantified by measuring the absorbance at 405 nm with an ELISA reader and was compared with a standard melanin curve.[9]

Inhibitory activity on tyrosinase and melanin production in mouse B16F10 melanoma cells with α-MSH – A stimulant, melanocyte stimulating hormone (α-MSH) (Sigma, St. Louis, MO, USA) was used to know influence of samples to tyrosinase activity and melanin production on hyperstimulation B16F10 melanoma cells. These experiments were tested in the same way (inhibitory activity on melanin production in mouse B16F10 melanoma cells) with presence of 100nM α-MSH.[9]

Result and Discussion

Activity guided isolation – Chromatographic isolation of fraction 3 and 4 of the Phragmitis Rhizoma has led to the isolation four phenolic compounds. Structures of compounds **1 - 4** (Fig. 1A) were identified as methyl gallate,[10] *p*-hydroxy cinnamic acid,[11] (+)-lyoniresinol,[12] and (+)-lyoniresinol-9'-*O*-*β*-D-glucopyranoside[13] respectively, by comparing the spectral (MS, NMR) data with the values reported in the literatures. Compounds **3** and **4** were

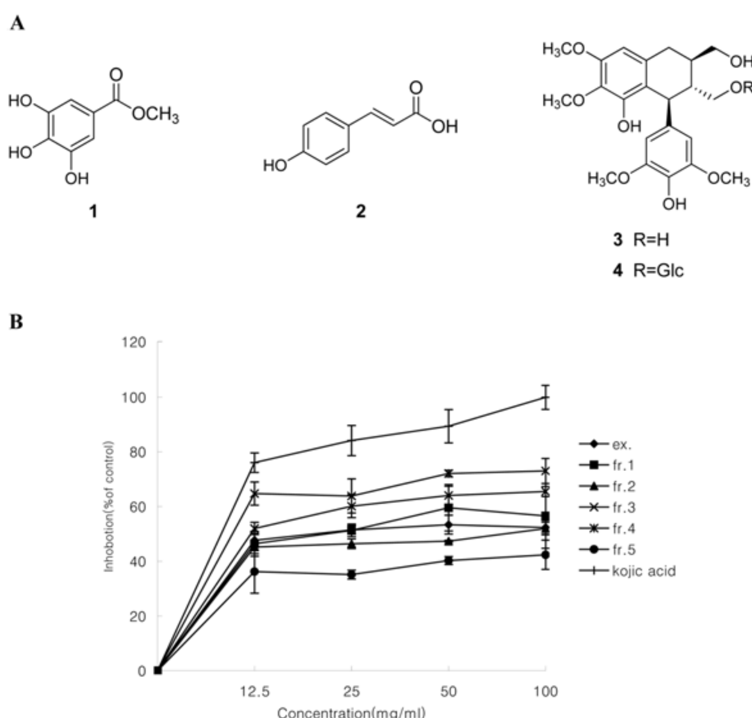

Fig. 1. Structures of compounds **1 - 4** (A) and inhibitory effects of extract and fractions **1 - 5** on tyrosinase activity (B). Results were expressed as % of control absorbance and data were mean ± S.D. of at least three experiments.

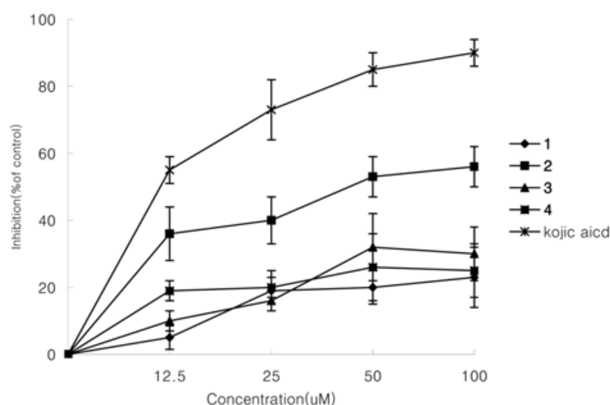

Fig. 2. Inhibitory effects of compounds **1** - **4** on tyrosinase activity. Results were expressed as % of control absorbance and data were mean ± S.D. of at least three experiments.

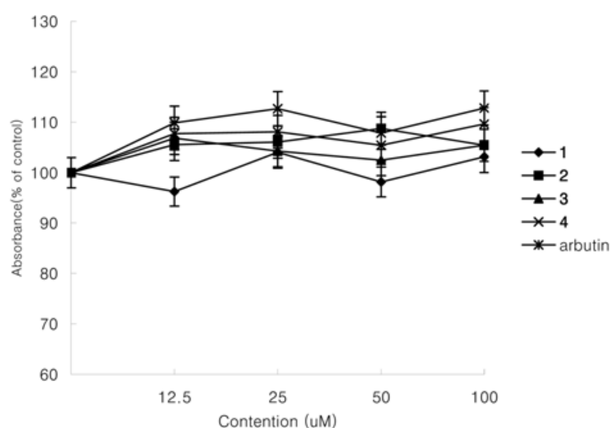

Fig. 3. Effects of compounds **1** - **4** on the viabilities of B16F10 melanoma cells. The viability of the cells was measured by MTT assays. Results were expressed as % of control absorbance and data were mean ± S.D. of at least three experiments.

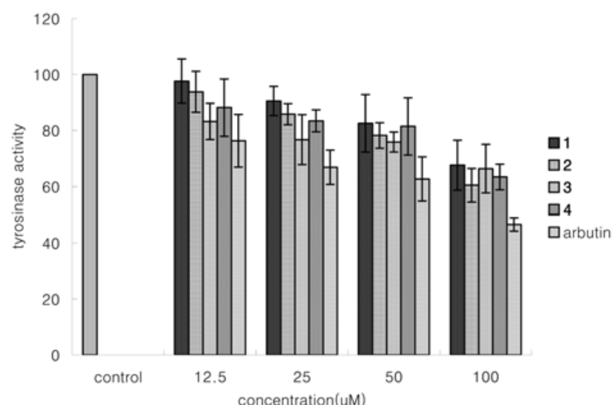

Fig. 4. Inhibitory effects of compounds **1** - **4** on tyrosinase activities in B16F10 melanoma cells. Tyrosinase activities were measured at 492 nm. Results were expressed as % control and data mean ± S.D. of at least three experiments.

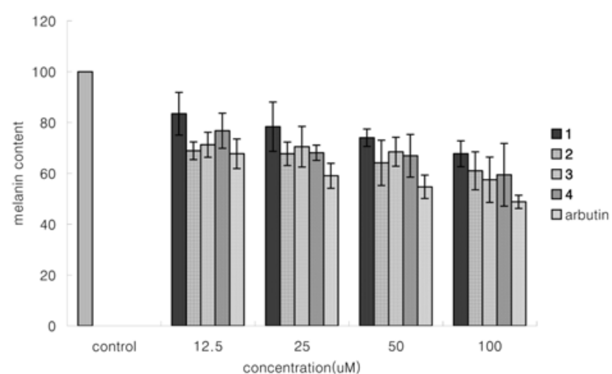

Fig. 5. Inhibitory effects of compounds **1** - **4** on melanin synthesis in B16F10 melanoma cells. Melanin contents were measured at 405 nm. Results were expressed as % control and data mean ± S.D. of at least three experiments.

isolated first from Phragmitis Rhizoma.

Among four isolated compounds **(1 - 4)**, compound **2** reduced the activity of tyrosinase moderately compared with positive control, kojic acid (Fig. 2.).

MTT assay in mouse B16F10 melanoma cells – Before the determination whitening activity in mouse B16F10 melanoma cells, MTT assays were performed to test cytotoxicity of the isolated compounds **(1 - 4)** from Phragmitis Rhizoma. Each compound from Phragmitis Rhizoma exhibited no cytotoxicity at experimental doses compared with blank. These results demonstrated that whitening activity of each compound from Phragmitis Rhizoma were not owing unrelated to its cytotoxicity (Fig. 3.).

Inhibitory activity on tyrosinase and melanin production in mouse B16F10 melanoma cells – Inhibitory activities on tyrosinase and melanin production were determined with treatment of each compound in B16F10 melanoma cells. As concentration increases, all compounds reduced moderately on tyrosinase activity and melanin production compared with positive control, arbutin ($p < 0.05$) (Fig. 4. and 5.). Especially, compound **2** and **3** showed more potent activities than other in compounds inhibitory activity on tyrosinase of B16F10 melanoma cells related to their own actions.

Inhibitory activity on tyrosinase and melanin production in mouse B16F10 melanoma cells with the α-MSH – The α-MSH was known as melanogenesis stimulant which raised intracellular cyclic adenosine monophosphate (c-AMP). Tyrosinase activity and melanin production were increased effectively by the treatment of 100 nm α-MSH. And as the concentration of compounds were increased the tyrosinase activity and melanin production were reduced compared with positive control, arbutin ($p < 0.05$**)** (Fig. 6. and 7). It seems that all com-

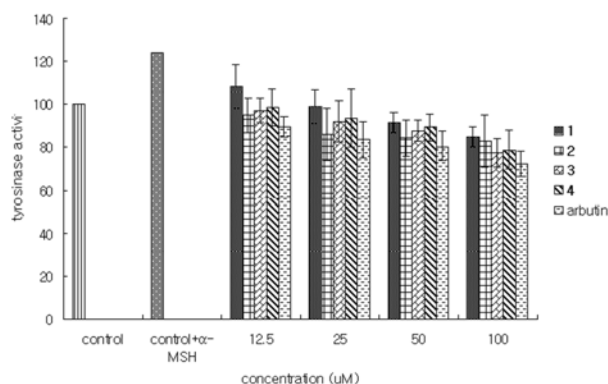

Fig. 6. Inhibitory effects of compounds **1** - **4** on melanin synthesis in the presence of 100 mM α-MSH in B16F10 melanoma cells. Tyrosinase activities were measured at 405 nm. Results were expressed as % control and data mean ± S.D. of at least three experiments.

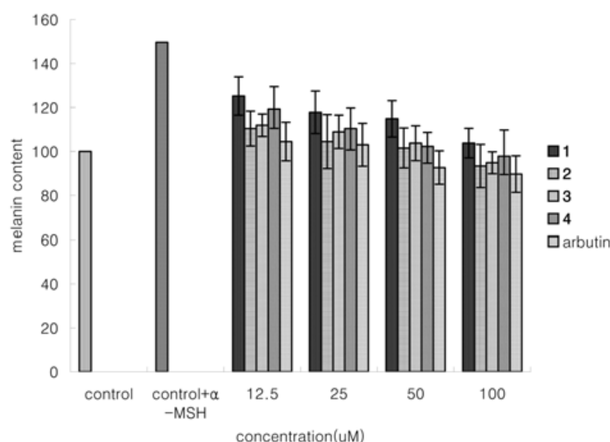

Fig. 7. Inhibitory effects of compounds **1** - **4** on melanin synthesis in the presence of 100 mM α-MSH in B16F10 melanoma cells. Melanin contents were measured at 405 nm. Results were expressed as % control and data mean ± S.D. of at least three experiments.

pounds inhibit the over-melanization of B16F10 melanoma cells related to c-AMP dependent regulation. These results suggest that two phenolic acids and two lignans from the Phragmitis Rhizoma might be a potent inhibitor against melanogenesis and these compounds could be further developed as skin whitening cosmeceuticals.

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