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Extract of Enzyme-Hydrolyzed Green Tea Seed as Potent Melanin Synthesis Inhibitor

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Melanogenesis is the process of melanin synthesis and distribution by a cascade of enzymatic and chemical reactions in melanocytes.[1] The role of melanin is to protect the skin from the harmful effects of ultraviolet light and scavenging free radicals. However, increased levels of epidermal melanin synthesis can darken the skin and produce various dermatologic disorders, such as melasma, age spots or liver spots, and actinic damage.[2]

Recently, the application of naturally occurring products as melanin synthesis inhibitors in cosmetics has attracted much interest.[3] For example, plant polyphenols have been the target of several studies,[4] resulting in repeated reviews of their classification, occurrence, structural aspects, reactivity, biochemistry, and biogenesis. Extensive literature is available on the screening of tyrosinase inhibitors, among phenolics of plant origin, and polyphenols are currently the target of numerous studies.

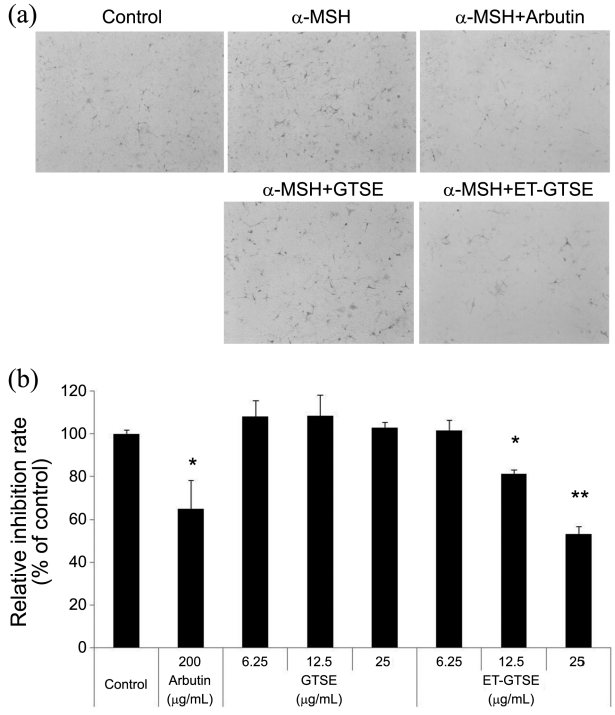


Figure 1. Inhibitory effect of ET-GTSE on melanin synthesis. (a) Effect of GTSE and ET-GTSE on melanin synthesis in melan-a/SP-1 co-culture (b) Melanin synthesis inhibition activity of GTSE and ET-GTSE (*P < 0.01; **P < 0.001: statistically significant versus value of control group.)

Tea [Camellia sinensis (L.) O. Kuntze, Theaceae] has been cultivated widely in Asia for centuries. Recently, as the popularity of green tea has increased, the production of green tea seed (GTS) has also increased. GTS contains many biologically active compounds such as saponins, flavonoids, vitamins, and oils.[5] It exhibited a broad spectrum of biological activities such as antitumor activities and weight reducing activity.[6-8]

Several studies have reported the potential of enzyme treatments for enhancing the activity levels and number of antioxidants in order to increase the content of functional components in food products.[9,10] We previously reported that the several kaempferol glycosides can be produced from GTS extract (GTSE) by enzymatic hydrolysis. However, the depigmentation effect of GTS by enzyme treatment was not studied. In the present study, tyrosinase and melanin synthesis inhibitory effect of GTSE was investigated before and after subjecting the extract to enzymatic hydrolysis. Enzyme treatment was applied to GTSE for 1 h with stirring at 27 °C. After incubation, each aliquot was extracted with ethanol. Prepared samples were evaluated for melanin synthesis activity.

Figure 1 shows inhibitory effect of GTSE and Enzyme treated GTS extract (ET-GTSE) on melanin synthesis. ET-GTSE reduced α-Melanocyte-stimulating hormone (α-MSH) stimulated mouse melanocyte/keratinocyte (melan-a/SP-1) co-culture assay in Figure 1(a). The melanin contents of melan-a cells also were suppressed by treatment of ET-GTSE in a dose-dependent manner in Figure 1(b). However, GTSE showed no inhibitory activity at tested concentration. To investigate the possible mechanisms of ET-GTSE underlying the inhibition of melanogenesis, the effects of the ET-GTSE on tyrosinase inhibition activity as well as the expression of tyrosinase were examined. Tyrosinase inhibitory activity was evaluated with mushroom tyrosinase (Table 1). GTSE

Table 1. Inhibition of GTSE, ET-GTSE on mushroom tyrosinase activity

Compound	Tyrosinase IC_{50}[a] (μg/mL)
GTSE	> 100
ET-GTSE	97.10 ± 5.7

[a]Values were determined from linear concentration – inhibition curves and are the means of three experiments.

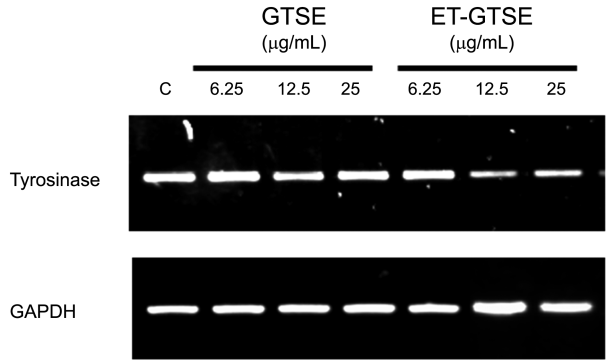


Figure 2. Inhibitory effect of ET-GTSE on tyrosinase gene expression on melan-a cells.

showed no inhibitory activity at tested concentrations (IC_{50} > 100 μg/mL). However, ET-GTSE showed inhibitory activity (IC_{50} = 97.10 ± 5.7 μg/mL).

Figure 2 shows effect of ET-GTSE on tyrosinase gene expression on melan-a cells. ET-GTSE suppressed the expression of tyrosinase. Tyrosinase is particularly crucial to melanogenesis. Direct inhibition of the activities of these enzymes could be useful in preventing skin hyper-pigmentation. These results indicated that enzymatic hydrolysis onto GTSE could change the composition of its contents and this change resulted in tyrosinase and melanin synthesis inhibitory activities.

The changes of contents are shown in Figure 3. The identification of compounds was carried out by ^{1}H and ^{13}C NMR studies. All signals in ^{1}H NMR and ^{13}C NMR spectra of compounds are in good agreement with those published previously.[9,11] Compounds **1** and **2** are camelliaside A and B from GTSE in Figure 3(a) and compounds **3** and **4** are nictoflorin and kaempferol from ET-GTSE in Figure 3(b). The structure of each compounds isolated from GTSE and

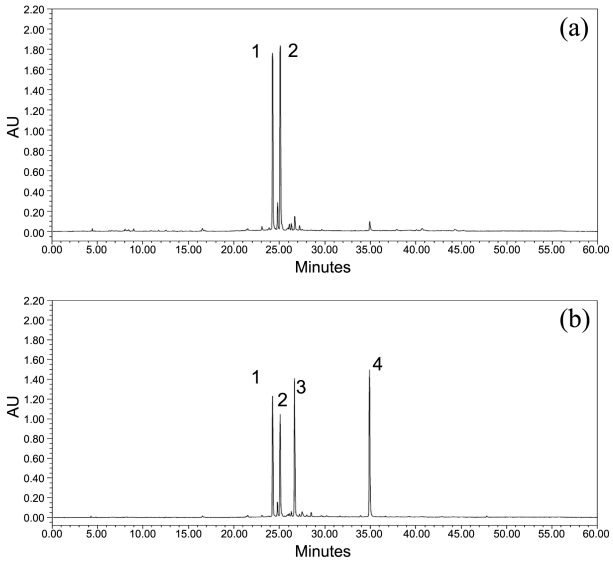
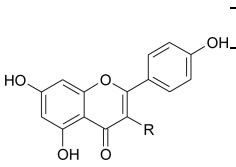


Figure 3. HPLC profiles of (a) GTSE and (b) ET-GTSE. Key to peak identity: 1. Camelliaside A 2. Camelliaside B 3. Nictoflorin 4. Kaempferol.



Compound	R
1	Gal(1→2)[Rha(1→6)]Glc
2	Xyl(1→2)[Rha(1→6)]Glc
3	Rha(1→6)Glc
4	H

Figure 4. Structure of compounds isolated from ET-GTSE.

ET-GTSE shows in Figure 4. From HPLC results, Contents of nictoflorin (3) and kaempferol (4) was significantly increased by enzymatic hydrolysis (Table 2). We hypothesize that tyrosinase and melanin synthesis inhibitory activity may have originated from nictoflorin and kaempferol after enzyme treatment of GTSE.

In order to confirm our hypothesis, Tyrosinase inhibitory activities of deglycosylation products and its substrate, camelliaside A (1) and camelliaside B (2), were evaluated. The results are summarized in Table 3. Specifically, kaempferol (4) exhibited inhibitory activities with IC_{50} values of 151.40 ± 0.7 μM. However, camelliaside A (1) and camelliaside B (2) showed no inhibitory activity. The weak inhibitory activity was shown in nictoflorin (3). nictoflorin (3) showed 20.1% inhibition of tyrosinase at 500 μM concentration.

The tyrosinase catalyzes the oxidation of L-3,4-dihydroxyphenylalanine (L-Dopa) to ortho-dopaquinone, and antioxidants may prevent the oxidation step by tyrosinase reaction in melanogenesis.[12] We measured antioxidation effect of deglycosylation products and its substrate, camelliaside A (1) and camelliaside B (2), by superoxide radical scavenging effect. This test is a non-enzymatic method currently used to provide basic information on the reactivity of compounds to scavenge free radicals.

Table 4 shows the superoxide radical scavenging activity of compounds **1** to **4**. Nictoflorin (3) and kaempferol (4)

Table 2. Contents of compounds **1**, **2**, **3**, and **4** in GTSE and ET-GTSE

Compound	Content (mg/g)	
	GTSE	ET-GTSE
1	53.5	18.3
2	56.1	16.2
3	1.5	19.8
4	0.7	15.1

Table 3. Tyrosinase inhibitory and Melanin synthesis inhibition activities of kaempferol and its glycosides

Compound	Tyrosinase IC_{50}^{a} (μM)	Melanin synthesis IC_{50}^{a} (μM)
1	> 500	> 500
2	> 500	> 500
3	> 500 (20.1 %)[b]	30.45 ± 1.2
4	151.40 ± 0.7	39.99 ± 0.8
Arbutin	330.26 ± 0.4	98.05 ± 0.4

[a]Values were determined from linear concentration-inhibition curves and are the means of three experiments. [b]Inhibitory activity (% of control) at 500 μM.

Table 4. Superoxide radical scavenging activity of kaempferol and its glycosides

Compound	IC_{50} (μM)[a]
1	137.44 ± 1.2
2	68.42 ± 2.5
3	83.53 ± 0.9
4	92.42 ± 4.4
L-Ascorbic acid	73.13 ± 7.0

[a]Values were determined from linear concentration-inhibition curves and are the means of three experiments.

showed significant antioxidant activity with IC_{50} values of 83.53 ± 0.90 and 92.42 ± 4.41 μM, respectively. This result shows that the inhibitory effect of kaempferol (3) and nictoflorin (4) in ET-GTSE against tyrosinase reaction is evidence for direct correlation with antioxidant activity.

To evaluate the inhibitory potency against the melanin synthesis, compounds **1** to **4** were assessed for their inhibitory effect in the cultured melan-a cells. Among those compounds, Nictoflorin (IC_{50} = 30.45 ± 1.2 μM) and kaempferol (IC_{50} = 39.99 ± 0.8 μM) significantly suppressed the cellular melanin synthesis as compared to the inhibitory activity of arbutin (IC_{50} = 98.05 ± 0.4 μM) as shown in Table 3, whereas camelliaside A and B showed low inhibition activity. From the above data, it is suggested that the number of the glycosyl group is important in order to exhibit anti-melanin synthesis activity. Nictoflorin has a diglycosyl group and kaempferol has no glycosyl group in C-3 positions. This makes them potential candidates for melanin synthesis inhibition reaction, whereas the triglycosyl group in C-3 position reduced inhibition activity (camelliaside A and B).

Compound **4** is due to the direct tyrosinase inhibition binding of dihydroxyl group to the copper ion like arbutin.[16] The activity of compound **3** (nictoflrorin), which has low tyrosinase inhibition and high antioxidation activity, has diglycosyl group. It has another mechanism of melanin synthesis inhibition such as tyrosinase glycosylation inhibition and cell growth suppression.[17,18] These results suggest that ET-GTSE with Compounds **3** and **4** can be utilized for the development of new candidate for tyrosinase and melanin synthesis inhibitors.

Experimental

Preparation of GTSE. Green tea seed (C. sinensis (L.) O. Kuntze, Theaceae) cultivated in the reclaimed land in Jeju island, S. Korea, was harvested. Green tea seed were sun dried and grinded to powder by a grinder machine in the laboratory. The powdered sample (100 g) was suspended in 1 L of 70% ethanol (v/v) and kept overnight in a shaker at room temperature. The extracts were filtered through Advantec 5B Tokyo Roshi Kaisha Ltd, Japan. The ethanol extract was dried using a vacuum rotary evaporator (EYLA N-1000, Tokyo, Japan) in a 50 °C water bath. Dried samples were weighed and kept at 4 °C for further analysis.

Preparation of ET-GTSE. GTSE (0.5 g) in 8 mL of 0.02 M sodium-acetate buffer (pH 5.0) with 2 mL of cellulase (Sigma Chemical, USA) solutions was incubated with stirring at 27 °C for a fixed time. The amount of each enzyme was adjusted to provide a final concentration in the mixture of 100 units/g of GTSE. Each sample and blank was used as a reaction control. All samples were prepared in duplicate. After incubation, each aliquot (200 μL) was extracted with ethanol (800 μL) and centrifuged at 12000 rpm (4 °C) for 10 min. Supernatants were transferred to new tubes and dried completely *in vacuo*. It was stored frozen for analysis. The analysis of GTSE and ET-GTSE was was carried out by the following HPLC methods. The HPLC system consisted of a Waters 2695 separation module and a 2996 photodiode array (PDA) detector. A 250 mm × 4.6 mm i.d. Mightysil C18 reverse phase column (Kanto Chemical, Japan) was employed. The detector wavelength was set at 263 nm. The mobile phase used for the analysis of samples was a mixture of distilled water (A) and acetonitrile (B) with gradient elution. The gradient elution was 15-80% B in 60 min at a flow rate of 1 mL/min.

Melan-a/SP-1 Co-culture. Melan-a cells (kindly provided from Dr. Bennett) were seeded in 6-well plate at 4.8×10^4 cells/well in RPMI 1640 (Gibco, USA). After 24 h, the plates were washed with Dulbeco's Phosphate buffered saline (DPBS) twice to eliminate traces of phorbol myristate acetate (PMA), and standard keratinocyte medium (SKM) was added. After 24 h incubation, SP-1 keratinocytes (purchased from ATCC) were seeded in each well containing the melanocytes at 2.4×10^4 cells/well with SKM. One and three days later, fresh SKM containing 200 nM α-MSH and sample was added. After treatment, the cultures phenazine methosulphate (PMS)were photographed.

RNA Isolation and RT-PCR. Melan-a cell cultures were washed twice with DPBS, and were lysed using Trizol (Invitrogen, CA, USA) by repetitive pipetting and samples were incubated for 30 min at room temperature before chloroform addition and samples centrifugation (12000 rpm, 15 min, 4 °C). The aqueous phase was then tranferred to fresh tubes and isopropanol added. The supernatant was incubated for 10 min at room temperature before centrifugation (12000 rpm, 10 min, 4 °C). RNA pellets were washed with 75% ethanol and re-extracted with RNeasy kit (Qiagen, CA, USA). The RNA yield was estimated by determining the optical density at 260 nm. Subsequently, cDNA was synthesized from total RNA with reverse transcriptase (Invitrogen, CA, USA). After reverse-transcription, polymerase chain reaction (PCR) was performed, and gene expression level was examined by gel-eletrophoresis.

Measurement of Mushroom Tyrosinase. Mushroom tyrosinase and L-tyrosine were purchased from Sigma Chemical. The reaction mixture for mushroom tyrosinase activity consisted of 150 μL of 0.1 M phosphate buffer (pH 6.5), 3 μL of sample solution, 8 μL of mushroom tyrosinase (2100 unit/mL, 0.05 M phosphate buffer at pH 6.5), and 36 μL of 1.5 mM L-tyrosine. Tyrosinase activity was determined by reading the optical density at 490 nm on a microplate reader (Bio-Rad 3550, Richmond, CA, USA) after incubation for

20 min at 37 °C.

Superoxide Radical Scavenging Assay. Superoxide scavenging activities of the compounds were determined by monitoring the competition of those with nitroblue tetrazolium (NBT) for the superoxide anion generated by the PMS-NADH system by Liu *et al.*[13] Superoxide radicals were generated in 1 mL 20 mM Tris-HCl buffer pH 8.0 containing 0.05 mM NBT, 0.01 mM phenazine methosulphate (PMS) and test compounds were preincubated for 2 min. The reaction was initiated by the addition of 0.078 mM NADH. Blue chromogen, formed due to NBT reduction, was read at 560 nm.

Melanin Synthesis Assay. The melanin content for inhibition assay on melanin synthesis was measured using a modified method.[14] After incubation for 5 days with the compounds, the cells were washed with ice-cold PBS (pH 7.4) and the pellets were collected by trypsinization and centrifugation. The pellets were dissolved in 1 N NaOH solution and the melanin was measured for absorbance at 400 nm using an ELISA reader. The melanin content was determined in μg from a synthetic melanin standard curve and correlated to mg protein using method of Kalb and Bernlohr.[15]

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