Melanin synthesis and skin wrinkle inhibitory effects of the medicinal mushroom *Ganoderma applanatum*

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ABSTRACT: Anti-melanogenesis and skin anti-wrinkle effects of methanol (ME) and hot water (HE) extracts from the fruiting bodies of *Ganoderma applanatum* were investigated in this study. The total phenolic contents of the ME and HE of the mushroom were 11.68 and 3.15 µg GAEs/mg, respectively, whereas the total flavonoid contents of the ME and HE were 21.82 and 2.69 µg QEs/mg, respectively. The survival rate of B16-F10 murine melanoma cells treated with 750 µg ME and HE were 83.46% and 85.54%, respectively, thereby suggesting that mushroom extracts were slightly cytotoxic at the tested concentration. The *in vitro* tyrosinase inhibition by ME (83.15%) and HE (83.44%) was significantly lower than that of kojic acid (99.61%), the positive control, at 2.0 mg/mL. Although the inhibition of cellular melanin synthesis in B16-F10 melanoma cells by 2.0 mg/mL of ME (50.24%) and HE (51.24%) was lower than that of arbutin (64.84%), the inhibition by both ME and HE was higher than 50%. Collagenase inhibition by HE was comparable to 2.0 mg/mL epigallocatechin (EGCG), the positive control; however, elastase inhibition by ME and HE was lower than that of EGCG at the concentration tested. The results showed that the fruiting bodies of *G. applanatum* had good anti-tyrosinase, good anti-collagenase, and moderate anti-elastase activities, which might be useful for developing novel skin-whitening and anti-wrinkle agents.

KEYWORDS: Anti-melanogenesis, Anti-wrinkle, Cytotoxicity, *Ganoderma applanatum*

INTRODUCTION

Tyrosinase is one of the key enzymes responsible for melanin biosynthesis, catalyzing the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and converting DOPA to dopaquinone (Zawistowski *et al*., 1991). Melanin is a colored pigment, which plays a key role in protecting the skin from harmful ultraviolet (UV) light. Skin hyperpigmentation, resulting from increased melanin-synthesizing enzyme activity caused by excessive

exposure to UV radiation may be responsible for skin disorders, including age spots, freckles, melasma, and malignant melanoma (Robb, 1984). Recently, tyrosinase inhibitors have become important treatments to prevent hyperpigmentation and other melanin-related disorders. Since light-colored skin is preferred by some people, the cosmetic industry has turned its attention to tyrosinase inhibitors to treat skin hyperpigmentation (Brenner and Hearing, 2008). To address these problems, natural compounds derived from plants and microorganisms, including kojic acid, arbutin, azelaic acid, gentisic acid, and others, have also been developed (Chang, 2009). The effectiveness of depigmentation agents is determined by mushroom tyrosinase inhibition in cell-free systems or by the inhibition of cellular tyrosinase and new tyrosine inhibitors from natural sources are continually being screened.

Skin aging is a complex biological process of living organisms influenced by both intrinsic and extrinsic factors that include hormonal changes, metabolic process, inheritance, cigarette smoking, and UV radiation. These factors can cause changes in skin thickness, loss of skin tone, and wrinkles (Zhang and Duan, 2018). Skin is composed of epidermal, dermal, and subcutaneous tissue

and the soft outermost part of the skin is an extracellular matrix (ECM) consisting of collagen, fibronectin, and elastin (Muiznieks and Keeley, 2013). Collagen contributes flexibility, strength, and elasticity to the skin. It also preserves the structural integrity of the skin and plays a key role in normal cellular functions during skin morphogenesis (Shoulders and Raines, 2009). Elastin is a crucial protein existing in the connective tissue of the ECM that contributes elasticity and three-dimensional integrity to the skin. Collagenase, a zinc-dependent endopeptidase, breaks down peptide bonds, specifically ECM type 1 collagen. Elastase, a proteolytic enzyme, which degrades collagen and elastin, also regulates the mechanical and structural integrity of the ECM connective tissue (Kim *et al*., 2004). Therefore, there is increasing interest in natural products that can halt and delay skin aging, skin sagging, and wrinkling.

Mushrooms have been used worldwide as a good source of food and folk medicines for thousands of years. The fruiting bodies of mushrooms possess biologically beneficial metabolites, such as polysaccharides, polyphenols, flavonoids, terpenoids, and other compounds, with good medicinal qualities that contribute to immune potentiation, as well as antitumor, anti-hyperglycemic, anti-hyperlipidemic, and anti-inflammatory activities (Lindequist *et al*., 2005; Wasser and Weis, 1999). Wu *et al*. (2016) reported that many mushrooms have known to possess ingredients such as phenolics, flavonoids, terpenoids, polysaccharides, vitamins, and volatile organic compounds. These compounds are beneficial to anti-aging, anti-wrinkle, skin whitening, and good moisturizing effects, which make them good candidates for cosmetics materials. Lee *et al*. (2016) demonstrated that hot water extract from fruiting bodies of *Tremella fuciformis* exhibited the anti-melanogenesis effect by decreasing melanin contents and tyrosinase activity in B16-F10 melanoma cells. Furthermore, the extract showed anti-wrinkle effect by increasing the synthesis of type I procollagen and reduced mRNA expression of matrix metalloproteinase 1 (MMP-1) activity in the human dermal fibroblast, which suggested that hot water extract of *T. fuciformis* reduce the cellular melanogenesis expression and protect against wrinkles caused by ageing, and UV radiation damage.

G. applanatum, known as an artist's conk mushroom, belongs to the phylum Basidiomycota, class Agaricomycetes, order Polyporales, and family Ganodermataceae, and is distributed worldwide (Park and Lee, 2003). Several mushroom species belonging to the genus *Ganoderma* have been studied for their potential medicinal antioxidant, anti-diabetic, anti-hyperlipidemic, anti-microbial, and antitumor activities (Ying *et al*., 1987). Although a few species of *Ganoderma* have been used in Asian countries for thousands of years for their medicinal properties, only *G. lucidum* has been cultivated on an industrial scale and utilized for medicinal purposes for human health. However, there are only a few published reports on the medicinal effects of *G. applanatum* (Tsivileva *et al*., 2018).

Therefore, the aim of this study was to evaluate the anti-melanogenesis, and anti-wrinkle effects of methanol and hot water extracts from the fruiting body powder of *G. applanatum*.

MATERIALS AND METHODS

Chemicals and reagents

Dimethyl sulfoxide, (DMSO), tyrosinase, L-3,4-dihydroxyphenylalanine (DOPA), kojic acid, synthetic melanin, arbutin, collagenase, and porcine pancreatic elastase were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). All other chemicals and solvents used for the study were of analytical grade.

Mushroom extract

The fruiting bodies of *G. applanatum* were collected from Incheon City Grand Park and identified by Dr. Kyung-Rim Lee, mycologist. The voucher specimen (IUM 3393) was deposited in the "Culture Collection of Mushrooms", Incheon National University (Fig. 1). The fruiting bodies were air-dried (45°C for 48 h) and finely pulverized. To prepare the methanol extract (ME), 20 g of the powder and 400 mL of 80% methanol were

Fig. 1. Fruiting body of *Ganoderma applanatum* used in this study.

mixed in an orbital shaker (125 rpm) for 24 h at 25°C. The solution was filtered through filter paper. To obtain the hot water extract (HE), the same amount of mushroom powder was boiled for 3 h in 400 mL of deionized distilled water and filtered. Then, the first ME and HE residues were extracted with 400 mL of 80% methanol or hot water, respectively, twice, using the same method described above. The extracts were evaporated to dryness at 40°C and remaining water and solvent were removed with freeze-drier.

Total phenolics and flavonoid contents

The total phenolic contents of fruiting bodies of *G. applanatum* were determined by the Folin–Ciocalteu method (Meda *et al.*, 2005). Briefly, 0.1 g of methanol and hot water extracts of the mushroom dissolved in 1 mL deionized water. The solution (0.1 mL) was mixed with 2.8 mL of deionized water, 2 mL of 2% sodium carbonate (Na_2CO_3), and 0.1 mL of 50% Folin–Ciocalteau reagent. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750 nm against a deionized water blank on a spectrophotometer. Gallic acid (GA) was used as a positive control. the data were expressed as microgram (μg) gallic acid equivalents (GAE)/mg of lyophilized powder. The data were expressed as microgram (μg) gallic acid equivalents (GAE)/mg.

The total flavonoid content was determined by the aluminum chloride colorimetric method described by Chang *et al.* (2002) , Briefly, 0.1 g of methanol and hot water extracts were dissolved in 1 mL deionized water. The solution (0.5 mL)was mixed with 1.5 mL of 95% alcohol, 0.1 mL of 10% aluminum chloride hexahydrate ($AlCl_3$), 0.1 mL of 1 M potassium acetate (CH_3COOK), and 2.8 mL of deionized water. After incubation at room temperature for 40 min, the absorbance of reaction mixture was measured at 415 nm against deionized water blank on a spectrophotometer. Quercetin was used as a positive control.

Cytotoxicity assay
Cell culture

B16-F10 murine melanoma cells were purchased from the Korean Cell Line Bank of the Korean Cell Line Research Foundation in Seoul, Korea. The B16-F10 melanoma cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL),

streptomycin (100 μg/mL), and 1% L-glutamine at 37°C with 5% atmospheric CO_2 in a humidified incubator.

Cell viability test

The effect of ME and HE on B16-F10 melanoma cell viability was evaluated by the MTT assay (Mosmann, 1983). The cells (5×10^4) were incubated in a 96-well plate for 24 h at 37°C. Then, the cells were treated with various concentrations of ME and HE (25-750 μg/mg) for the B16-F10 melanoma cells and incubated for 48 h in MTT solution (200 μL of 0.5 mg/mL) was added to each well and the plate was incubated for 4 h in the dark. The resulting colored formazan crystals were dissolved in 200 μL of dimethyl sulfoxide (DMSO) and the absorbance of the solution was measured at 570 nm with a microplate reader.

Inhibitory effect of melanin synthesis
Inhibition of tyrosinase

Inhibition of tyrosinase by the mushroom extracts was determined using a published method (Kim and Ryu, 2012) with slight modification. Briefly, the reaction mixtures were prepared by the addition of 40 μL of 31 units/mL tyrosinase to 40 μL of varying concentrations of ME and HE (0.125-2.0 mg/mL), 40 μL of 1.5 mM L-tyrosine, and 80 μL of 0.1 M phosphate buffer (pH 6.8). Then, the mixture was incubated for 10 min at 37°C. The absorbance was measured with a microplate reader at 475 nm. Tyrosinase inhibition was calculated using the following formula:

$$\text{Inhibition of tyrosinase (\%)} = [(Abs_c - Abs_s)/Abs_c] \times 100$$

where Abs_c represents the absorbance of the vehicle control and Abs_s represents the absorbance of the test sample. BHT was used as a reference standard.

Inhibition of L-DOPA autooxidation

The inhibition of L-DOPA autooxidation by ME and HE was performed using a slight modification of a published method (Masuda *et al.*, 2005). Briefly, the reaction mixtures were prepared by the addition of 40 μL of 31 units/mL tyrosinase to 40 μL of varying concentrations of ME and HE (0.125-2.0 mg/mL) and incubated for 10 min at 37°C. Then, the reaction was initiated by adding 40 μL of 2.5 mM L-DOPA solution in phosphate buffer and 80 μL of 0.1 M phosphate

buffer (pH 6.8) and incubated for 10 min at 37°C in the dark. The absorbance of the solution was measured at 475 nm with a microplate reader. The percentage of L-DOPA autooxidation inhibition was calculated using the following formula:

Inhibition of DOPA autooxidation (%) = [(Abs$_c$ − Abs$_s$)/Abs$_c$] × 100

where Abs$_c$ represents the absorbance of the vehicle control and Abs$_s$ represents the absorbance of the test sample. Kojic acid was used as a reference standard.

Inhibition of cellular melanin synthesis

The melanin content of B16-F10 melanoma cells treated with varying concentrations of mushroom extracts was performed using the method published by Hosoi *et al.* (1985). The cells ($4 × 10^4$ cells/well) were cultured in a 96-well plate in DMEM. After 24 h of incubation, the cells were treated with ME and HE (25-500 µg/mL) for 72 h containing 0.4 µM melanocyte-stimulating hormone (MSH). The cells were harvested by the addition of 300 µL of 1% Triton X-100 (in PBS), centrifuged for 5 min at 3,000 rpm, and the supernatant removed. Then, 100 µL of 1N NaOH and 200 µL of 10% DMSO were added to the cell pellets and kept for 1 h at 60°C. The absorbance of the solution was measured using a microplate reader at 405 nm and compared to standard curves of synthetic melanin. Arbutin was used as a reference standard.

Skin anti-wrinkle effect
Inhibitory effect of elastase

The elastase inhibitory effect of ME and HE was assessed by previously reported method (Kim *et al.*, 2004). Porcine pancreatic elastase (PE-EC. 3.4.21.36) was dissolved in sterile water to make a 3.33 mg/mL stock solution. The substrate, N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (AAAPVN), was dissolved in 0.2 M Tris-HCl buffer (pH 8.0). The reaction was carried out in a 96-well microplate. Each well contained 120 µL of 0.2 M Tris-HCl buffer, 50 µL mushroom extract (0.125 − 2.0 mg/mL) in Tris-HCl buffer, 30 µL PE, and 50 µL of 1.6 mM AAAPVN. The extract was preincubated at 25°C for 15 min before the addition of the substrate. The absorbance was measured with a microplate reader at 402 nm. The percent inhibition of elastase was calculated as follows:

Elastase inhibition (%) = [(Abs$_c$ − Abs$_s$)/Abs$_c$] × 100 where Abs$_c$ represents the absorbance of the vehicle control and Abs$_s$ represents the absorbance of the test sample. EGCG was used as a reference standard and distilled water was used as a negative control.

Inhibitory effect of collagenase

The inhibition of collagenase by ME and HE was investigated using previously reported method (Van Wart and Steinbrink, 1981) with some modifications. Collagenase from *Clostridium histolyticum* (ChC - EC 3.4.24.3) was solubilized in the 50 mM of 1.44 units/mL Tricine buffer (pH 7.8). The substrate, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg trifluoroacetate salt (PZ-peptide), was dissolved in 2.5 mM tricine buffer. Twenty-five microliters of mushroom extracts (0.125-2.0 mg/mL) were incubated with 20 µL of enzyme in 155 µL buffer for 15 min at 37°C before addition of 50 µL PZ-peptide to initiate the reaction and the mixture was incubated for 60 min at 37°C. The reaction was terminated by adding 1 mL of citric acid (3%). Then, 5 mL of ethyl acetate was added and the solution was vigorously shaken and allowed to stand for 10 minutes. The absorbance of the supernatant was measured at 320 nm using a microplate reader. EGCG was used as a reference standard. The inhibition of collagenase was calculated using the following formula:

Collagenase inhibition (%) = [(Abs$_c$ − Abs$_s$)/Abs$_c$] × 100

where Abs$_c$ represents the absorbance of the vehicle control and Abs$_s$ represents the absorbance of the test sample.

Statistical Analysis

All data were expressed as mean ± standard deviations (SD) and SPSS V.13 (SPSS Inc., Chiago, IL, USA) was used for statistical analysis. One-way analysis of variance followed by Tukey multiple comparisons were used to compare means between groups. Differences between means at the 5% ($p \leq 0.05$) level were considered statistically significant.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents
The results showed that the total phenolic and flavonoid

Table 1. Total phenolics and flavonoid contents of methanol and hot-water extract from fruiting bodies of *Ganoderma applanatum*

Samples	Yields (% w/w)	Phenolics content (µg GAEs/mg) extract	Flavonoid content (µg QEs/mg) extract
Ganoderma applanatum Methanolic extraction	3.31	11.68 ± 0.08	21.82 ± 0.1
Ganoderma applanatum Hot-water extraction	5.09	3.15 ± 0.16	2.69 ± 0.35

Results are expressed as means ± standard deviation (SD, n = 3) GAEs, gallic acid equivalents; QEs, quercetin evalents

contents in the metahnol and hot water extracts of *G. applanatum* varied considerably. Total phenolic contents from methanol extract and hot water extract of *G. applanatum* ranged from 11.68 ± 0.08 to 3.15 ± 0.16 µg GAE/mg, respectively (Table 1). This study showed that total phenolic contents in the methanol extract was higher than that of hot water extract.

The total flavonoid contents in the methanol and hot water extracts of *G. applanatum* ranged from 21.82 ± 0.10 to 2.69 ± 0.35 µg QEs/mg, respectively (Table 1), suggesting that extraction by methanol gave the higher phenolic and flavonoid contents as compared to the hot water extract. Nguyen *et al*. (2013) reported that phenolic contents of methanol extract from fruiting bodies of *Phellinus xeraticus* was 16.37 µg GAE/g, suggesting that phenolic contents of fruiting bodies from *P. xeranticu*s was higher compared to *G. applanatum*, whereas flavonoid content of *P. xeranticu*s was lower (2.40 µg QEs/mg) than that of *G. applanatum*. (3.15 µg QEs/mg).

Phenolics and flavonoids are well-known for possessing one or more aromatic rings with large numbers of hydroxyl groups, which benefit human health by scavenging free radicals and inhibiting hydrolytic enzyme and tyrosinase activity (Walter and Marchesan, 2011; Yoon *et al*., 2011). Many researchers have demonstrated a positive relationship between the concentrations of phenolic compounds and *in vitro* tyrosinase inhibition and cellular melanin synthesis inhibition activities in B16 mouse melanoma cells (Sun *et al*., 2017).

Cell viability

To determine the extracts from *G. applanatum* fruiting bodies exhibited a cytotoxic effect on B16-F10 melanoma cells, the cells were treated with varying concentrations of ME and HE for 72 h and the cell viability was determined by the MTT assay. The cell viability of the B16-F10 melanoma cells treated with ME and HE ranged from 108.60 to 83.45% and from 111.80 to 88.54%, respectively, at 50 to 750 µg/mL (Fig. 2).

The cell viabilities of the B16-F10 melanoma cells decreased with increasing concentrations of the extracts. Since the viabilities of B16-F10 melanoma cells treated with 750 µg/mL ME and HE demonstrated 83.46% and
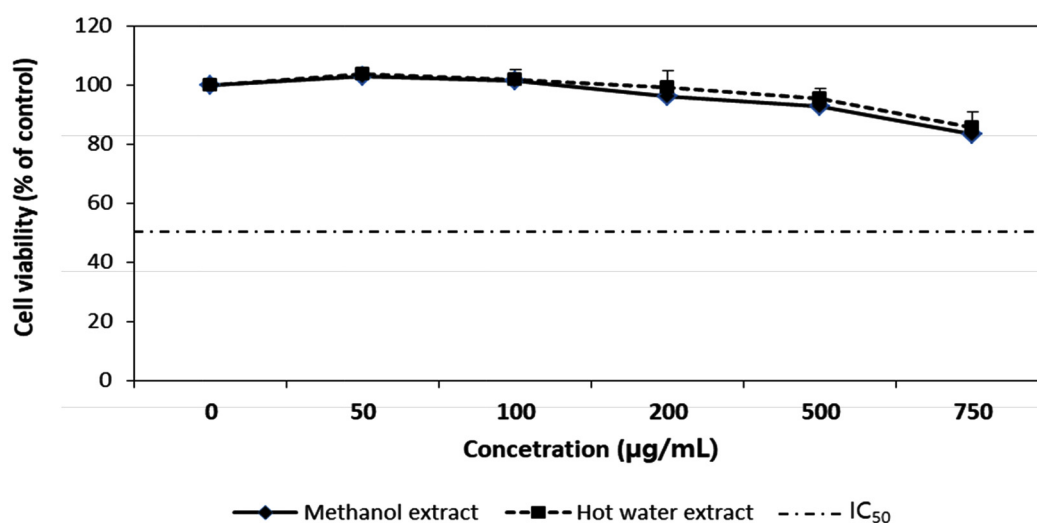

Fig. 2. Cytotoxic effects of methanol and hot water extracts from fruiting bodies of *Ganoderma applanatum* on B16-F10 melanoma cells. The results are expressed as means ± SD (n = 3).
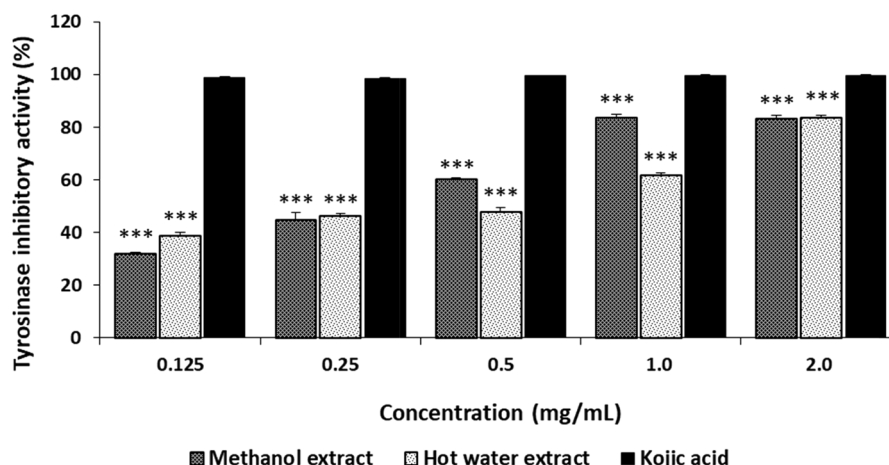
Fig. 3. Tyrosinase inhibitory activities of methanol and hot water extracts from fruiting bodies of *Ganoderma applanatum*. Results are expressed as means ± standard deviation (SD, n=4). $^{***}p < 0.001$ *vs.* Kojic acid group.

85.54% viabilities, respectively, suggesting that 750 μg/mL ME and HE of *G. applanatum* were slghtly cytotoxic to the B16-F10 melanoma cells. Therefore, the inhibition of cellular tyrosinase and melanin synthesis in B16-F10 melanoma cells were analyzed at 50-750 μg/mL mushroom extracts.

Inhibitory effect of melanogenesis
Inhibition of tyrosinase

The tyrosinase inhibition by ME and HE from the fruiting bodies of *G. applanatum* ranged from 31.88% to 83.15% and from 38.88% to 83.44%, respectively, at 0.125-2.0 mg/mL (Fig. 3) and increased with increasing concentrations. However, kojic acid, the reference standard, demonstrated excellent tyrosinase inhibition of 98.74 to 99.61% at 0.125-2.0 mg/mL. These results suggest that ME and HE exhibited a moderate inhibitory effect at the concentrations tested. Alam *et al*. (2011) reported that the tyrosinase inhibition by methanol and hot water extracts from the fruiting bodies of *P. salmoneostramineus* ranged from 20.90% to 63.29% and from 15.25% to 49.25%, respectively, at 0.125-1.0 mg/mL, demonstrating greater tyrosinase inhibition by *G. applanatum* than by *P. salmoneostramineus*.

Phenolic compounds are aromatic compounds containing phenolic rings and carboxylic acid. Flavonoids are a group of polyphenolic compounds found in many plant and mushroom species. Polyphenols are regarded as substrates for tyrosinase and are the largest group of tyrosinase inhibitors. Several polyphenols were shown to inhibit tyrosinase by binding to the active site of the enzyme,

resulting in irreversible inactivation of the enzyme by a substrate-like interaction (Wang *et al*, 2014). Antioxidant activity may also be one of the important mechanisms of tyrosinase inhibition. Some flavonoids like quercetin, naringin, and kaempferol also inhibit tyrosinase by chelating the copper ions in tyrosinase (Kim *et al*., 2006).

The inhibitory effect of ME and HE toward tyrosinase might be due to the polyphenolic compounds and flavonoids present in the mushroom extracts.

Inhibition of L-DOPA auto-oxidation

L-DOPA is one of the precursors of melanin during melanogenesis. L-DOPA is synthesized from L-tyrosinase by tyrosinase hydroxylase. Tyrosinase can also convert L-DOPA to dopaquinone, eventually leading to melanin. The *in vitro* inhibition of L-DOPA autooxidation by *G. applanatum* is summarized in Fig. 4. The inhibition of L-DOPA autooxidation by ME and HE ranged from 38.25% to 78.46% and from 29.68% to 44.77%, respectively, at 0.125 to 2.0 mg/mL, whereas kojic acid demonstrated excellent L-DOPA inhibition of 86.14 to 98.72% at the concentration tested. The results showed that ME and HE moderately inhibited L-DOPA autooxidation. The inhibition of L-DOPA autooxidation by ME and HE, however, was less when L-DOPA was used as a substrate than when L-tyrosine was used as a substrate of tyrosinase. Since tyrosinase uses two different binding sites for tyrosine hydroxylase and L-DOPA oxidase (Hwang and Lee, 2007), our experimental results suggest the selective effectiveness of tyrosinase for the two different substrates of the mushroom during melanogenesis.
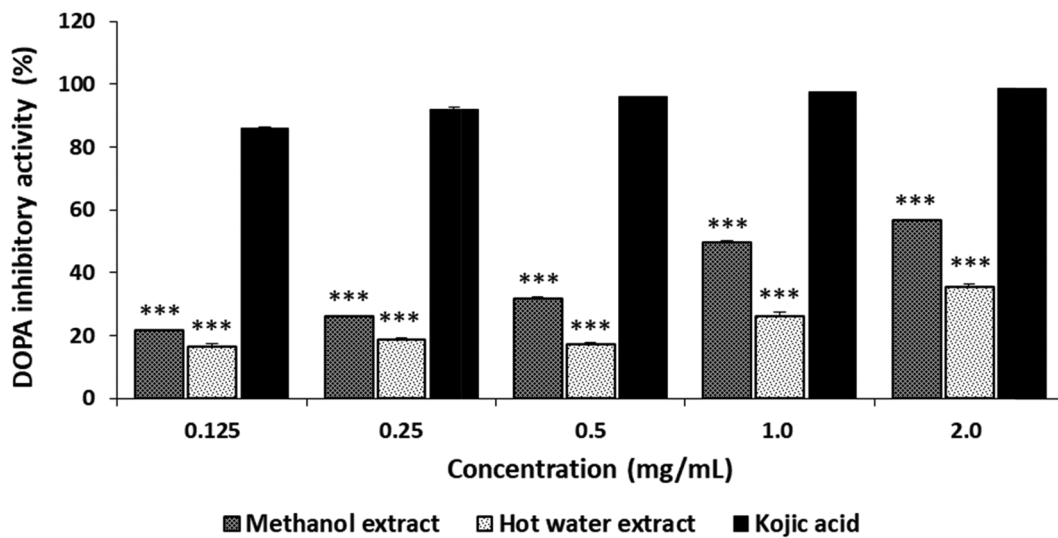
Fig. 4. DOPA auto-oxidation inhibitory activities of methanol and hot water extracts from fruiting bodies of *Ganoderma applanatum*. Results are expressed as means ± standard deviation (SD, n=4). ***$p < 0.001$ *vs.* Kojic acid group.

Inhibition of cellular melanin synthesis

To confirm whether the moderate cellular tyrosinase inhibition detected in the previous experiment was related to the inhibition of melanin synthesis in B16-F10 melanoma cells, melanin synthesis in the melanoma cells was further investigated. The inhibition of melanin production in the B16-F10 melanoma cells treated with ME and HE ranged from 18.74% to 50.24% and from 26.09% to 51.23%, respectively, at 25-500 µg/mL, whereas inhibition of melanin synthesis treated with arbutin on B16-F10 cells ranged from 26.5% to 64.84% at the concentration tested (Fig. 5). The results indicated that the inhibition of melanin synthesis by arbutin was

greater than those of ME and HE. Although the inhibition of melanin synthesis in B16-F10 melanoma cells by ME and HE was significantly lower than that of arbutin, the inhibition of melanin synthesis by ME and HE at 500 µg/mL was greater than 50%. Increased concentrations of the mushroom extracts resulted in decresed cellular melanin synthesis in tthe B16-F10 melanoma cells.

Chen *et al.* (2021) reported that culture broth and mycelia extracted with 50% ethanol from medicinal mushroom *Antrodia cinnamomea* exhibited 32% reduction of melanin production in B16-F10 melanoma cells at 200 µg/mL, indicating that inhibition of melanin synthesis in
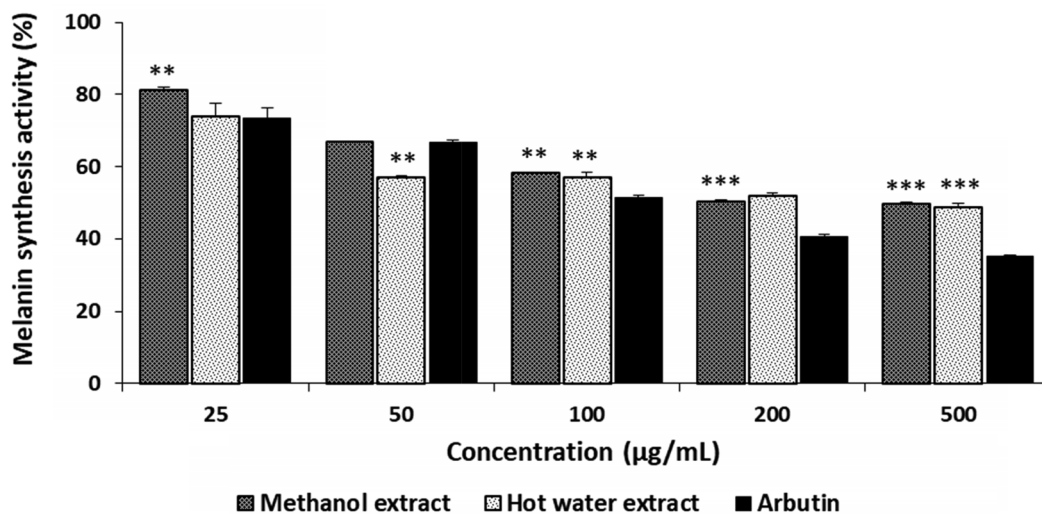

Fig. 5. Melanin synthesis activities of methanol and hot water extracts from fruiting bodies of *Ganoderma applanatum* in B16-F10 melanoma cells. Results are expressed as means ± standard deviation (SD, n=4). ***$p < 0.001$, **$p < 0.01$ *vs.* Arbutin group.
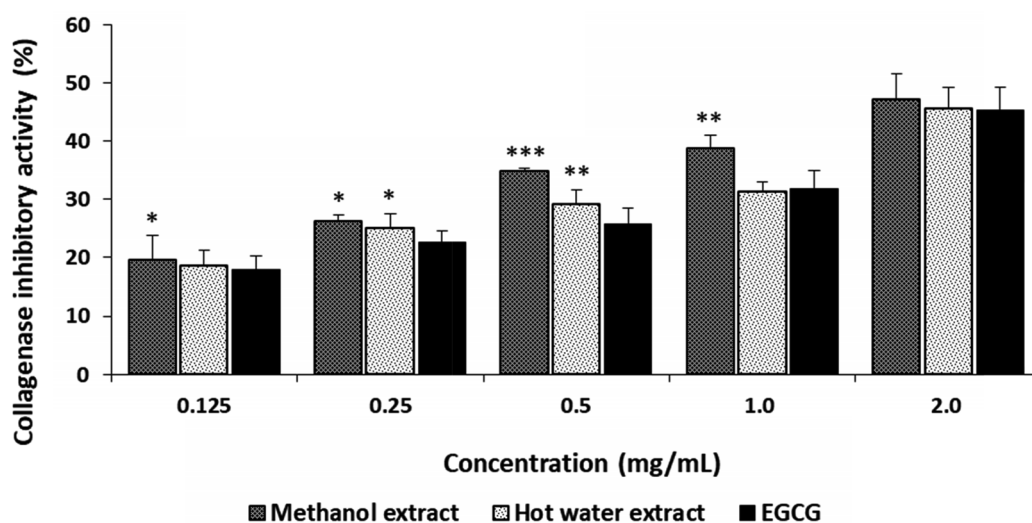
Fig. 6. Collagenase inhibitory activities of methanol and hot water extracts from fruiting bodies of *Ganoderma applanatum*. Results are expressed as means ± standard deviation (SD, n=4). EGCG, epigallocathechin-3-gallate. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs*. EGCG group.

the melanoma cells of the ME (49.58%) and HE (47.09%) was higher than that of the *Antrodia cinnamomea* ethanol extract at the same concentration tested. The results demonstrated that the mushroom extracts, not only inhibited *in vitro* tyrosinase activity and L-DOPA autooxidation, but also melanin synthesis in B16-F10 melanoma cells. Taken together, our findings suggest that the ME and HE from *G. applanatum* fruiting bodies may be regarded as potential novel anti-melanogenesis sources which can protect skin pigmentation from hazardous ultraviolet radiation.

Skin anti-wrinkle activity
Inhibitory effect of elastase

Elastase, a protease, is responsible for the breakdown of elastin in the ECM. Elastases can break down elastin, as well as other substrates, including collagen, fibronectin, and ECM proteins. Under natural conditions, elastase activity is crucial for the degradation of foreign proteins delivered through wounds (Tyagi and Simonn, 1993). To slow down the visible effects of skin aging, such as wrinkles, sagging, and freckles, the degradation of ECM-related proteins must be blocked, thereby terminating the loss of skin elasticity. The inhibition of elastase by ME and HE from the fruiting bodies of *G. applanatum* ranged from 35.59% to 69.29% and from 32.81% to 62.82%, respectively, at 0.125-2.0 mg/mL, whereas EGCG demonstrated elastase inhibitions of 80.92 to 92.27% at the concentration tested (Fig. 6). Therefore, the elastase inhibition by ME and

HE might be considered moderate compared to EGCG. Kim *et al*. (2014) reported that mycelial extract of *Tricholoma matsutake* demonstrated elastase inhibition of 81.4% at 100 μg/mL, indicating that the inhibitory effects of ME and HE from *G. applanatum* on elastase were lower than that of the *T. matsutake* extract. Choi and Lee (2016) demonstrated that the elastase inhibition by methanol and hot water extracts from the fruiting bodies of *Coriolus versicolor* ranged from 21.5% to 35.47% and from 17.57% to 25.86% at 1.0 to 3.0 mg/ mL, respectively, indicating that the elastase inhibition by ME and HE was greater than that of the *C. versicolor* fruiting bodies. Thus, the results suggest that the mushroom extracts blocked elastin and collagen from degradation and may represent potential anti-wrinkle agents.

Inhibitory effect of collagenase

Collagenases are metalloproteinases capable of cleaving various molecules found within the cells and ECM, including collagen, elastin, fibronectin, gelatin, and laminin. The bacterial collagenase (*Clostridium histolyticum*, ChC) used in this experiment also degrades proteins in the ECM (Pruteanu *et al*., 2011). Therefore, the bacterial collagenase was used to test the anti-collagenase activity of the mushroom extracts. The collagenase inhibition by the ME and HE from the fruiting bodies of *G. applanatum* ranged from 19.62% to 47.20% and from 18.53% to 45.56% at 0.125-2.0 mg/mL,
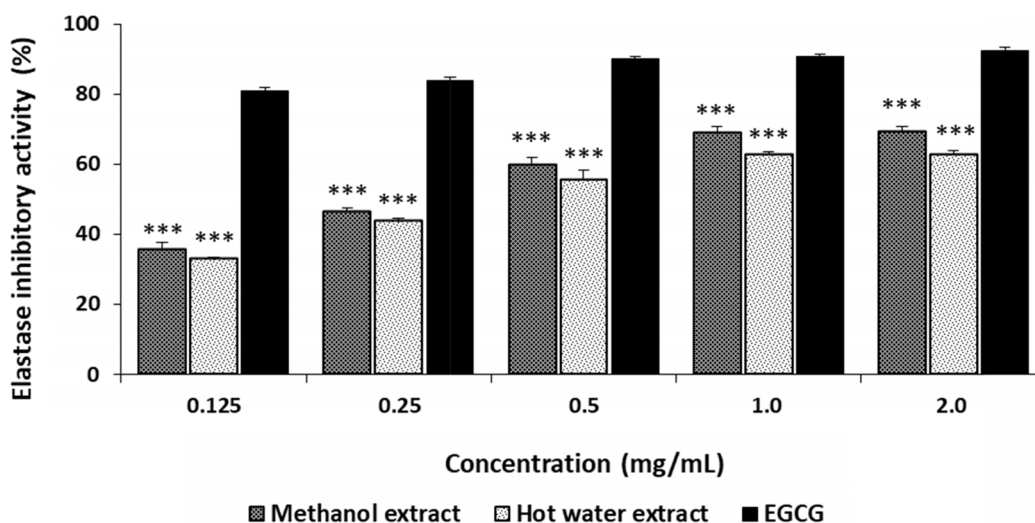
Fig. 7. Elastase inhibitory activities of methanol and hot water extracts from fruiting bodies of *Ganoderma applanatum*. Results are expressed as means ± standard deviation (SD, n=4). EGCG, epigallocathechin-3-gallate. [***]$p < 0.001$, [**]$p < 0.01$, [*]$p < 0.05$ *vs.* EGCG group.

respectively (Fig. 7), while EGCG inhibited collagenase 17.93 to 45.31% at the concentration tested. The results suggest that ME was an excellent inhibitor, whereas HE revealed good inhibitory effects comparable to EGCG at the concentrations tested. Cheon *et al*. (2008) reported that the collagenase inhibition by hot water, and ethanol extracts from fruiting bodies of *Phellinus linteus* ranged from 3.4% to 6.8% and from 33.4% to 89.6%, respectively, at 0.05-1.0 mg/mL, indicating that the collagenase inhibition by ME and HE from *G. applanatum* was higher than that of the water extract, whereas collagenase inhibition by fruiting body extracts of *G. applanatum* were lower than that of the ethanol extract from the *Ph. linteu* at 1.0 mg/mL. Since ME and HE exhibited good inhibitory effects on collagenase, the extracts may protect the ECM and collagen from degradation caused by photoaging due to UV light exposure. Although most elastase and collagenase inhibitory activities have been demonstrated in plants and microorganisms, including white tea, green tea, mulberry bark, ginkgo fruits, and mushrooms (Ghimeray *et al*., 2015; Melzig *et al*., 2001). This is the first report of elastase and collagenase inhibition by the fruiting body extracts from *G. applanatum* and may represent the source of a new anti-wrinkle agent.

In conclusion, the fruiting body extracts from *G. applanatum* exhibited good anti-tyrosinase, anti-melanin synthesis activities, as well as elastase and collagenase inhibition. The tyrosinase, and melanin synthesis inhibitory activities indicate that *G. applanatum* extracts might be new

natural sources for controlling inflammation and decreasing skin pigmentation. The inhibition of elastase and collagenase also suggest that the *G. applanatum* extracts might be good sources of compounds to treat skin wrinkle-related disorders. Additional research is necessary to verify the components providing the anti-tyrosinase, anti-melanin synthesis, and anti-wrinkle effects.

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