Jeju Inside Agency & Cosmetic Science Center[1], Department of Chemistry and Cosmetics, Jeju National University; Bio-Convergence Center[2], Jeju Technopark; CoseedBioPharm Co., Ltd.[3], Jeju, Republic of Korea

Anti-melanogenic effects of hot-water extracts from *Torreya nucifera* via MAPKs and cAMP signaling pathway on B16F10 cells

Ji-Hye Ko[1,#], Nari Lee[1,#], Hyun-Kyu Kang[1], Bong Seok Kim[2], Jung No Lee[3], Chang-Gu Hyun[1,*]

Received May 12, 2020, accepted June 19, 2020

**Corresponding author: C-G. Hyun, Department of Chemistry and Cosmetics, Jeju National University, Jeju, Republic of Korea*
cghyun@jejunu.ac.kr
[#]These authors contributed equally to this work.

Pharmazie 75: 565-570 (2020) *doi: 10.1691/ph.2020.0522*

Torreya nucifera is an evergreen tree in the family *Taxaceae*, the seeds, leaves, and stems of which have long been used as edible products and herbal medicines in Korea. Previous studies of biological activity have shown that *T. nucifera* has antioxidant and anti-inflammatory effects. However, the effect of *T. nucifera* leaves on melanogenesis are yet to be studied. In this investigation, we used B16F10 melanoma cells to test the efficacy of *T. nucifera* leaf hot water extract (TLWE). α-melanocyte stimulating hormone (α-MSH) stimulated B16F10 melanoma cells were treated with various concentrations of TLWE (50, 100, and 200 μg/mL). The results showed that TLWE reduced the melanin content and cellular tyrosinase activity in a concentration-dependent manner. It also inhibited the phosphorylation of p38 mitogen-activated protein kinase (p38) and c-Jun N-terminal kinase (JNK) in the mitogen-activated protein kinase (MAPK) signaling pathway. The compounds catechin and ρ-coumaric acid, which are known to have a whitening effect on skin, were detected by HPLC analysis. These results suggest that TLWE has an anti-melanogenic effect. In addition, the safety of TLWE was tested. The results of the skin irritation test showed that TLWE is harmless to the human skin, even at higher concentrations than those used in the experiment. Therefore, we suggest that the water extract of *T. nucifera* leaves has potential for use as a skin-whitening agent.

1. Introduction

The pigment melanin, produced by melanocytes in the epidermis, absorbs free radicals and disperses incoming ultraviolet rays, thus protecting the skin from harmful radiation and maintaining body temperature (Kim et al. 2004). When the skin is exposed to UVR, melanocytes and keratinocytes secrete α-MSH to promote the pathway for melanin biosynthesis (Friedmann et al. 1987). Increased α-MSH binds to the melanocyte-specific receptor MC1R and delivers a downstream signal, which activates adenylate cyclase to increase cAMP levels. Increased cAMP phosphorylates PKA and CREB to increase the expression of microphthalmia-associated transcription factor (MITF) (Lee et al. 2018; Kang et al. 2011).

MITF migrates into the nucleus and binds to the tyrosinase and TRP promoters to increase the expression of each of these genes. Enzymes, such as tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1), and tyrosine-related protein 2 (TRP-2), produced through this process are representative melanin synthesis-related enzymes (Bentley et al. 1994). In the early stages of melanogenesis, TYR plays a key role in promoting the hydroxylation of L-tyrosine to dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone. Next, DOPA quinone is spontaneously oxidized to form DOPA chrome. DOPA chrome tautomerase, also known as TRP-2, rearranges the DOPA chrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). DHICA is oxidized by TRP-1 to make indole-5,6-quinone carboxylic acid, which combine with each other to form melanin (Jiménez-Cervantes et al. 1994).

Serine/threonine family MAPKs are important factors in melanin biosynthesis, including p38, JNK, and ERK. MAPKs have been reported to be involved in the expression of MITF proteins. Phosphorylation of p38 and JNK inhibits the phosphorylation of MITF and induces its expression, to increase melanogenesis. ERK phosphorylates the serine73 portion of MITF, which degrades MITF and reduces melanogenesis (Chung et al. 2018).

An abnormally high level of melanin can cause hyperpigmentation, such as blemishes and freckles, which are considered, by some, to be cosmetic problems. Kojic acid, arbutin, hydroquinone, vitamin A, vitamin C, and their derivatives, are currently used in whitening cosmetic products (Kim et al. 2013). However, due to stability issues with these cosmetic formulations, in recent years, their use has been limited and consumers tend to prefer safer whitening cosmetics.

Torreya nucifera is a hardwood, evergreen, coniferous tree of the family *Taxaceae*. Its grain is straight, and it grows to 25 m in height and 2 m in diameter. It is endemic to the temperate forests of Southern Korea and Japan, growing at an altitude of less than 800 m. In particular, the 2,800 trees in the Gujwa-eup, Jeju Island, comprise the largest monospecies forest in the world, and are designated and protected as Natural Monument # 374. The wood has a high modulus of elasticity, is easy to process, and weathers well, so its uses include buildings, furniture, and ships. It is also widely used to make boards for the games Go and Shogi. Since ancient times, the seeds, leaves, and stems of *T. nucifera* trees have been used as edible and herbal ingredients (Choi et al. 2015). The seed coat from the mature seeds of the *T. nucifera* tree is called "visa;" it has pharmacological effects on digestion, constipation, and hemorrhoids and has also been used as an anthelmintic agent. *T. nucifera* has been previously reported to show pharmacological activity, including antioxidant, anti-inflammatory, hepatoprotective, and neuroprotective properties (Lee et al. 2017; Kim et al. 2016; Jeon et al. 2009; Kim et al. 2018).

However, the effect of *T. nucifera* leaves on melanin production has not been previously published. In this study, we investigated the effect of *T. nucifera* leaf extracts on B16F10 melanoma cells.

2. Investigations and results

2.1. Evaluation of the antioxidant activity of TLWE

The use of DPPH (2,2-diphenyl-1-picrylhydrazyl) to scavenge radicals is a relatively simple and widely used method for measuring antioxidant activity (Lee et al. 2018). It uses the principle that a relatively stable DPPH radical compound receives electrons or hydrogen from antioxidants, which reduce it to DPPH-H, thereby changing its color from purple to yellow.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) is also used to scavenge radicals in a similar way to DPPH. The DPPH method is mainly used to confirm the antioxidant capacity of a hydrophobic material, and the ABTS method is applicable to both hydrophilic and lipophilic material (Yu et al. 2002; Floegel et al. 2011).

All samples showed antioxidant activity, but it was confirmed that the efficacy of the hot water extract (TLWE) was higher than that of the 70 % EtOH extract and the EtOAc fraction (Table 1). As a result of measuring DPPH radical scavenging activity for TLWE, it was confirmed that the antioxidant activity increased in a concentration-dependent manner. At a concentration of 250 µg/mL, 84 % radical scavenging activity was observed. The $ABTS^+$ radical scavenging activity was also found to increase as a function of concentration. It showed an antioxidant efficacy of 96 % at 250 µg/mL. In both assays, above 250 µg/mL concentration, there was no significant increase anymore.

Table 1: IC_{50} values of *T. nucifera* leaf extract on DPPH and $ABTS^+$ radical scavenging activity

		Hot water Ext.	70% EtOH Ext.	EtOAc Fr.	Ascorbic acid
IC50 (µg/mL)	DPPH	53.10	55.26	63.96	6.62
	ABTS	16.57	21.89	25.21	2.22

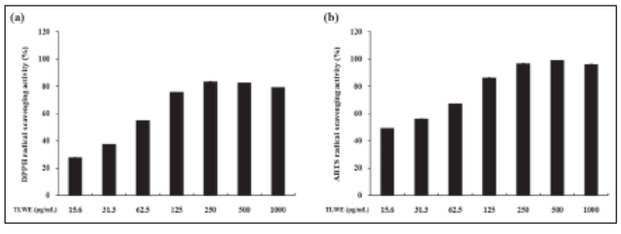

Fig. 1: Effects of TLWE on DPPH and $ABTS^+$ radical scavenging activity. TLWE (15.6, 31.3, 62.5, 125, 250, 500, and 1000 µg/mL) were reacted with the reagents DPPH or $ABTS^+$ for 15 min. The percentage of the control group was calculated and shown as a bar chart. Ascorbic acid was used as a positive control. Data are expressed as mean±standard deviation (SD) of at least four independent experiments. * IC_{50} values : (a) 53.10 µg/mL, (b) 16.57 µg/mL

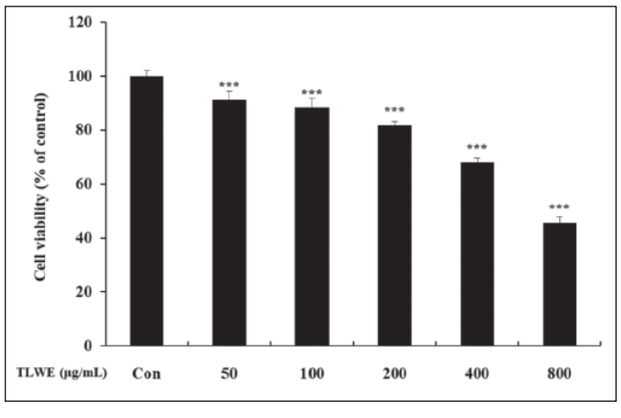

Fig. 2: Effects of TLWE on cell viability in B16F10 cells. The cells were treated with TLWE (50, 100, 200, 400, and 800 µg/mL) for 72 hr. The data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control).

2.2. Effects of TLWE on the viability of B16F10 cells

After culturing B16F10 cells for 72 h with TLWE (50, 100, 200, 400, and 800 µg/mL), cell viability was tested. The results are expressed as a percentage compared to the control (without TLWE). There was 82 % cell viability at a concentration of 200 µg/mL. Therefore, subsequent experiments were conducted at concentrations ≤ 200 µg/mL, which showed relatively low toxicity (Fig. 2).

2.3. Effects of TLWE on melanin production

B16F10 cells stimulated with α-MSH were treated with TLWE (50, 100, and 200 µg/mL) to confirm the effect on melanin production. α-MSH, used as a negative control, increased melanin production when compared to untreated control. However, when TLWE was used together with α-MSH, the melanin production was decreased in a concentration-dependent manner, and was found to be 42 % lower than that of the α-MSH-treated group, at 200 µg/mL (Fig. 3).

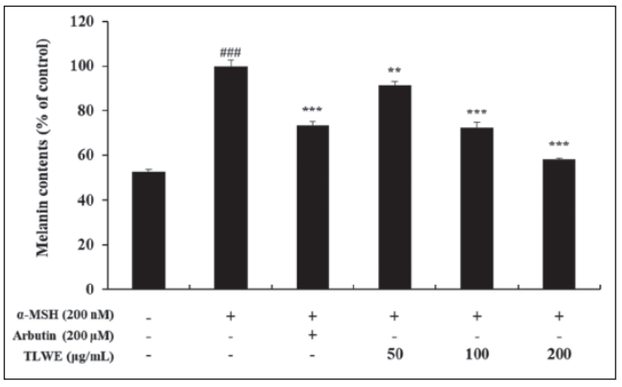

Fig. 3: Effects of TLWE on melanin production in B16F10 cells. The cells were treated with TLWE (50, 100, and 200 µg/mL) for 72 h. α-MSH (200 nM) was used as the negative control and arbutin (200 µM) was used as the positive control. The melanin content was expressed as a percentage compared to the untreated control. Data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. α-MSH, ### $p < 0.001$ vs. control).

2.4. Effects of TLWE on intracellular tyrosinase activity

α-MSH-stimulated B16F10 cells were treated with TLWE at a range of concentrations (50, 100, and 200 µg/mL) to investigate the effect on intercellular tyrosinase expression and activity. α-MSH, used as a negative control, increased tyrosinase activity by about 5-fold compared to the untreated control. However, when TLWE was treated together with α-MSH, it was confirmed that tyrosinase

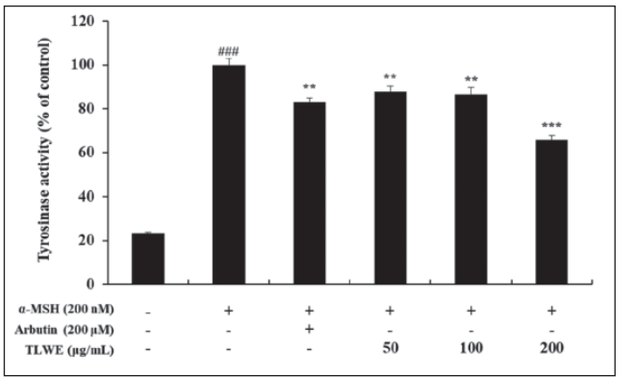

Fig. 4: Effects of TLWE on tyrosinase activity in B16F10 cells. The cells were treated with TLWE (50, 100, and 200 µg/mL) for 72 h. α-MSH (200 nM) was used as the negative control and arbutin (200 µM) was used as the positive control. Tyrosinase activity was expressed as a percentage compared to the untreated control. Data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. α-MSH, ### $p < 0.001$ vs. control).

activity decreased in a concentration-dependent manner (Fig. 4). At a concentration of 200 µg/mL, tyrosinase activity decreased by 34 % compared to that in the α-MSH-treated group.

2.5. Effects of TLWE on the expression of melanogenic enzymes in B16F10 cells

The previous results suggest that TLWE has the effect of inhibiting melanin production in B16F10 cells. Therefore, we tried to confirm this by western blot analysis.

The effect of TLWE was seen to affect the expression of tyrosinase, TRP-1, and TRP-2 proteins, which are enzymes related to melanin production (Fig. 5). For all three enzymes, the α-MSH treatment group showed increased protein expression, which was significantly decreased upon treatment with TLWE.

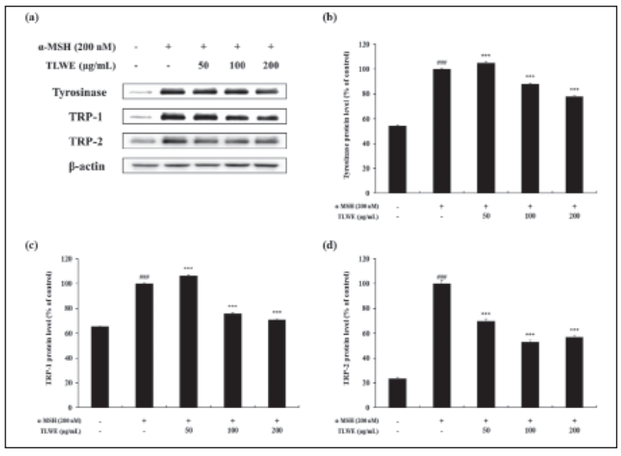

Fig. 5: Effects of TLWE on tyrosinase, TRP-1, and TRP-2 expression in B16F10 cells. The cells were treated with TLWE (50, 100, and 200 µg/mL) for 24 h. α-MSH (200 nM) was used as a negative control. (a) Protein levels were determined by western blotting. (b) Tyrosinase, (c) TRP-1, and (d) TRP-2 are expressed as a percentage compared to the value of β-actin. Data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. α-MSH, ### $p <$ 0.001 vs. control).

2.6. Effects of TLWE on the expression of MITF in B16F10 cells

MITF binds to the M-box of the tyrosinase gene promoter to increase the protein production of the melanin-producing enzymes tyrosinase, TRP-1, and TRP-2 (Vachtenheim et al. 2010). The effect of TLWE on the expression of MITF protein was investigated by western blot analysis.

As shown in Fig. 6, protein expression increased in the α-MSH-only treatment group but decreased significantly upon treatment with TLWE. These results indicate that TLWE inhibits the expression of melanin-producing enzymes in a concentration-dependent way.

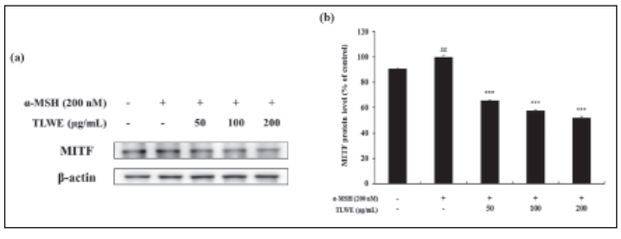

Fig. 6: Effects of TLWE on MITF expression in B16F10 cells. The cells were treated with TLWE (50, 100, and 200 µg/mL) for 22 h. α-MSH (200 nM) was used as a negative control. (a) Protein levels were determined by western blotting. (b) MITF was expressed as a percentage compared to the value of β-actin. Data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. α-MSH, ## $p < 0.01$ vs. control).

2.7. Effects of TLWE on MAPK signaling pathway in B16F10 cells

The effect of TLWE on the MAPK signaling pathway was investigated by western blot analysis. As shown in Fig. 7, the protein content of T-p38, T-JNK, and T-ERK was constant, regardless of TLWE treatment, but P-p38, P-JNK, and P-ERK showed changes. Phosphorylation of JNK, ERK, and p38 was decreased upon treatment with TLWE, compared to the α-MSH treatment group. According to previous studies, the phosphorylation of p38 and JNK inhibits the phosphorylation of MITF and induces its expression, thus increasing melanin production. The phosphorylation of ERK causes phosphorylation of the serine73 portion of MITF, degrading MITF and reducing melanin production (Ko et al. 2018)

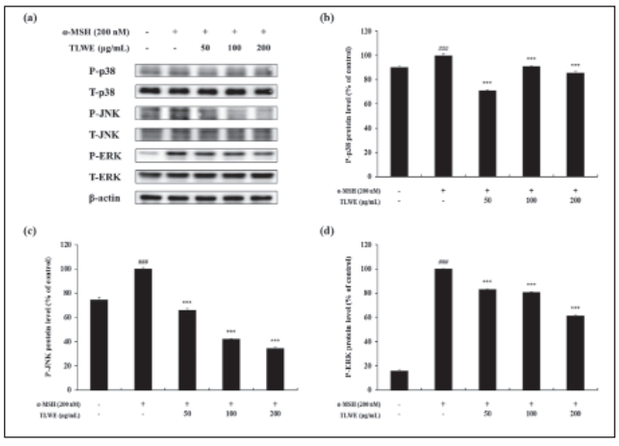

Fig. 7: Effects of TLWE on MAPK and β-actin expression in B16F10 cells. The cells were treated with TLWE (50, 100, and 200 µg/mL) for 15 min. α-MSH (200 nM) was used as the negative control. (a) Protein levels were determined by western blotting. (b) P-p38, (c) P-JNK, and (d) P-ERK were expressed as a percentage compared to the value of β-actin. Data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. α-MSH, ### $p < 0.001$ vs. control).

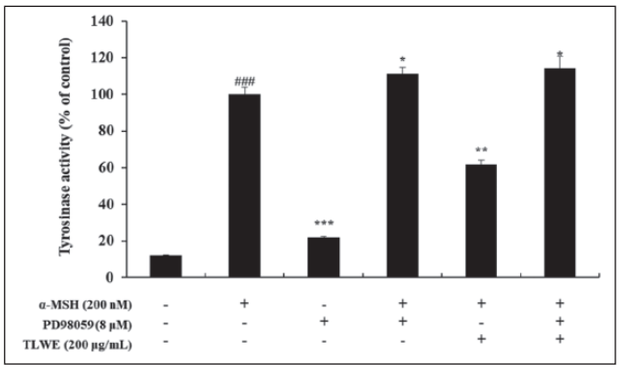

Fig. 8: Effects of TLWE and ERK inhibitor (PD98059) on tyrosinase activity in B16F10 cells. The cells were treated with TLWE (200 µg/mL) and PD98059 (8 µM) for 72 h. α-MSH (200 nM) was used as a negative control. Tyrosinase activity was expressed as a percentage compared to the untreated control. Data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. α-MSH, ### $p < 0.001$ vs. control).

However, our results suggest some differences from the mechanism of inhibition described previously. Therefore, to confirm the signal transduction pathway, tyrosinase activity was measured after treatment with an ERK phosphorylation inhibitor (PD98059). As shown in Fig. 8, the ERK phosphorylation inhibitor (PD98059) increased tyrosinase activity when treated alone or simultaneously with α-MSH. When TLWE was given to α-MSH-stimulated cells, the activity was decreased due to the effect of TLWE, but was increased when the inhibitor was treated simultaneously.

These results indicate that suppression of ERK phosphorylation induces *MITF* transcription, thereby increasing the expression of tyrosinase. Moreover, this suggests that the TLWE did not influence the ERK signaling pathway. The whitening effect of TLWE is, therefore, probably due to the effects of p38 and JNK phosphorylation and other signaling pathways.

2.8. Effects of TLWE on the cAMP signaling pathway in B16F10 cells

To investigate whether TLWE influences the cAMP pathway or another melanin-producing signaling pathway, tyrosinase activity was measured after treatment with AKT and PKA phosphorylation inhibitors, such as LY294002.

As shown in Fig. 9a, the AKT phosphorylation inhibitor LY294002 increased tyrosinase activity when used alone or simultaneously with α-MSH. When TLWE was applied to α-MSH-stimulated cells, the activity was decreased, due to the effect of TLWE, and was also slightly decreased when the inhibitor and TLWE were used simultaneously.

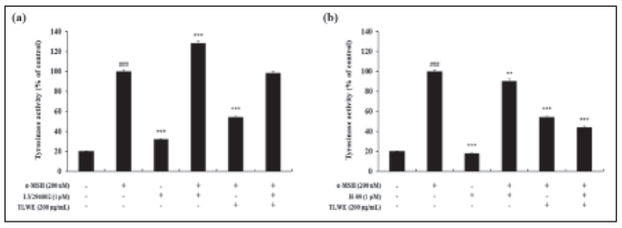

Fig. 9: Effects of TLWE and (a) AKT inhibitor (LY294002), (b) PKA inhibitor (H-89) on tyrosinase activity in B16F10 cells. The cells were treated with TLWE (200 μg/mL) and (a) LY294002 (1 μM), (b) H-89 (1 μM) for 72 h. α-MSH (200 nM) was used as a negative control. Tyrosinase activity was expressed as a percentage compared to the untreated control. Data are expressed as mean ± standard deviation (SD) of at least four independent experiments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. α-MSH, ### $p < 0.001$ vs. control).

However, since evidence from tyrosinase inhibition alone is insufficient for proof, the effect of TLWE was also sought on the PKA pathway, which is an AKT sub-mechanism.

PKA is known to induce the transcription of MITF through phosphorylation of CREB (Jian et al. 2011). Therefore, in order to investigate the effect of TLWE on the phosphorylation of PKA, tyrosinase activity was measured after treatment with a PKA phosphorylation inhibitor (H-89). From Fig. 9b, H-89 reduced the tyrosinase activity slightly when treated alone or simultaneously with α-MSH. When the cells were treated with TLWE, the activity was reduced, suggesting that TLWE affected the phosphorylation of PKA. Therefore, we suggest that TLWE regulates melanin production via the cAMP signaling pathways involved in AKT and PKA.

2.9. HPLC analysis of the indicator components of TLWE

HPLC analysis of the indicator component is a method that can be used to identify the active component in an extract. HPLC analysis was performed using catechin, which has been identified by previous studies as a constituent of *T. nucifera* leaf extract (Kim et al. 2010), and *p*-coumaric acid, a phenol compound commonly found in plants, as standards (An et al. 2008).

Catechin is a well-known flavonoid that is naturally distributed in various fruits, green tea, and red wine. Catechin derivatives have a wide range of biological effects, such as antioxidant and anti-inflammatory (Kim et al. 2014), and have been shown to enhance skin-whitening by inhibiting the expression of tyrosinase, an enzyme related to melanin production, in B16F10 cells (Sato et al. 2009). *p*-Coumaric acid is the most abundant of three isomers found in nature. It is structurally very similar to L-tyrosine (Fig. 10), and has been reported to inhibit melanin production by competing with L-tyrosine in B16F10 cells stimulated with α-MSH (An et al. 2010).

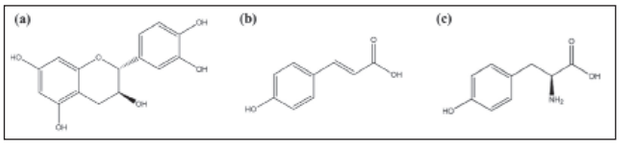

Fig. 10: Structures of (a) catechin, (b) *p*-coumaric acid, and (c) L-tyrosine.

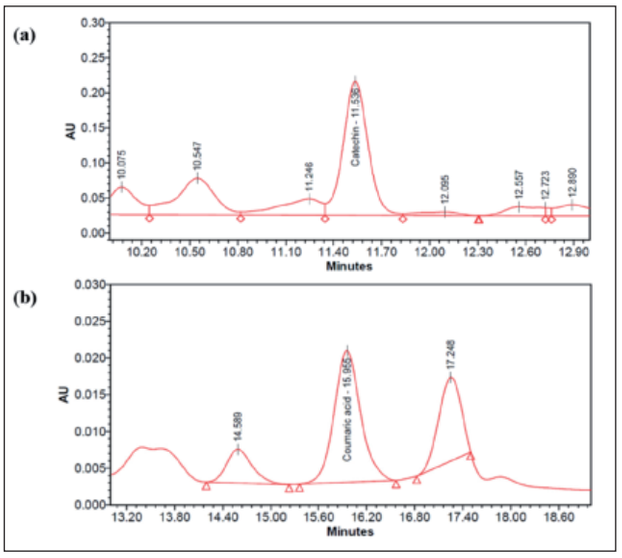

Fig. 11: HPLC chromatogram of TWLE. (a) Catechin, (b) *p*-coumaric acid.

As a result of HPLC analysis (Fig. 11), catechin (299.84 μg/mL) and *p*-coumaric acid (4.85 μg/mL) were both detected in TLWE (50 mg/mL). Therefore, it appears that TLWE inhibits melanin production due to the presence of these compounds, which could be responsible for its skin-whitening activity.

2.10. Human skin primary irritation test of TLWE

In order to evaluate whether TLWE could potentially be used as an ingredient in whitening cosmetics, a primary irritation test was conducted with a 24-h skin patch.

As shown in Table 2, TLWE did not cause any irritation (at 24, 48, and 72 h) 30 min after the patch was removed. As a result of calculating the irritation index, using the skin reaction score of each subject, TLWE obtained a skin irritation index of 0.011 at a concentration of 800 μg/mL (a concentration higher than the 200 μg/mL used in the previous experiment), therefore it was judged to be a nonirritating substance. In addition, the subjects did not show severe side effects, such as erythema, burning, or pruritus. These results suggest that TLWE is a safe material for application as a cosmetic ingredient.

Table 2: Skin primary irritation test results of TLWE

NO.	TLWE (800 μg/mL) Reaction			BLANK Reaction		
	24h*	48h	72h	24h*	48h	72h
Grade	1	0	0	1	0	0
Total number of observations	90			90		
Irritation Index	0.011			0.011		
Judgment	No irritancy			-		

* Measure at 30 minutes after removing the patch

3. Discussion

In this paper, research was conducted on a hot water extract of *T. nucifera* (L.) (TLWE), which has antioxidant activity and is associated with the inhibition of melanogenesis.

B16F10 cells were treated with TLWE (50, 100, 200, 400, and 800 μg/mL) to find the extent of toxicity, and subsequent experiments were conducted using a concentration of 200 μg/mL, which produced a cell viability of 82 %. The use of TLWE to treat cells stimulated with α-MSH confirmed the inhibitory effect on melanin production and tyrosinase expression and activity. In the case of the 70 % EtOH extract and the EtOAc fraction, which were simultaneously tested in advance, it was confirmed that tyrosinase activity was not significantly inhibited (data not shown). It was confirmed that the hot water extract showed better whitening activity. Therefore, further analysis was conducted with TLWE to find the mechanism of inhibition of melanin production.

As a result of western blotting, it was shown that TLWE inhibited the expression of tyrosinase, TRP-1, and TRP-2 proteins, which are enzymes related to melanin production, and the expression of MITF, a high regulatory factor, was also inhibited in a concentration-dependent manner.

The expression of MAPKs, involved in the regulation of MITF, was tested and confirmed, and TLWE was shown to decrease P-p38, P-JNK, and P-ERK. However, the mechanism of action cannot be confirmed by these results alone; therefore, tyrosinase activity was tested after treatment with inhibitors, to elucidate the signal transduction pathways.

It was found that TLWE did not influence ERK, in the MAPK signaling pathway, so the whitening effect of TLWE was expected to be due to the effects of p38 and JNK phosphorylation (Figs. 7-8). As shown in Fig. 9a, TLWE in α-MSH-stimulated cells inhibited tyrosinase activity with both AKT phosphorylation inhibitors and PKA phosphorylation inhibitors. In particular, TLWE was shown to affect the phosphorylation of PKA, leading us to conclude that TLWE regulates melanin production through cAMP signaling pathways, which are related to AKT and PKA.

HPLC analysis was then performed to identify the components causing the whitening activity of the TLWE extract. Contents were measured by selecting two indicators (catechin and *p*-coumaric acid) that have been reported to have whitening activity in previous studies. The content of catechin and *p*-coumaric acid in TLWE (50 mg/mL) was 299.83 μg/mL and 4.85 μg/mL, respectively, which suggests that these are the active ingredients in the inhibition of melanin production.

In addition, as a result of the skin primary irritation evaluation conducted by the Korea Dermatology Research Institute, TLWE was judged to be a nonirritating substance without side effects, even at a concentration of 800 μg/mL, which is 4-fold higher than the 200 μg/mL used in cell experiments.

Based on these results, TLWE can be said to have excellent antioxidant efficacy and inhibit melanogenesis by reducing melanin-producing enzymes by controlling MAPK and MITF pathways in B16F10 melanoma cells. Therefore, it is suggested that TLWE could be used as a skin-whitening agent that is safe for the human body.

4. Experimental

4.1. Chemicals and reagents

Torreya nucifera leaves in this study were purchased from Island Co., Ltd. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-ethylenediaminetetraacetic acid, and BCA protein assay kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). α-Melanocyte stimulating hormone (α-MSH), sodium hydroxide (NaOH), and protease inhibitor cocktails were purchased from Merck (Darmstadt, Hesse, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from VWR (Radnor, Pennsylvania, USA). Western blot experimental antibodies P-p38, T-p38, P-JNK, T-JNK, P-ERK, T-ERK, P-p105, T-p105, P-p65, T-p65, T-IκB-α, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA), and TRP-1, TRP-2, tyrosinase, and MITF were purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A). Dimethyl sulfoxide (DMSO), radioimmunoprecipitation (RIPA) buffer, enhanced chemiluminescence (ECL) kit, and 2X Laemmli sample buffer were purchased from Biosesang (Sungnam, Gyeonggi-do, Korea) and Bio-Rad (Hercules, CA, USA), respectively.

4.2. Extract preparation

The leaves of *Torreya nucifera* were separated from the branches and washed. Then, they were dried sufficiently in the shade and crushed into powder. The powder of *T. nucifera* leaves was mixed with hot water or 70 % EtOH, and fractionated with EtOAc to prepare three types of sample.

First, for hot water extraction, 101.76 g of powdered leaves was added to 1.8 L of deionized water, and the mixture was extracted while stirring with a magnetic bar at 90 °C for 6 h. Then, for 70 % EtOH extraction, 1040 g of powdered leaves were added to 3 L of 70 % EtOH, and the mixture was stirred with a magnetic bar at 25 °C. for 24 h. The powdered extracts obtained were fractionated using EtOAc. The extract was filtered through ADVANTEC No. 2 filter paper twice. The solvent was removed using a vacuum concentrator and lyophilized to obtain a sample in powder form.

The *T. nucifera* leaf hot water extract (TLWE) yield was 29.7 % and 70 % for EtOH extracts, and the EtOAc fraction yields were 16.29 % and 0.24 %, respectively. The extracts were stored at 4 °C before use.

4.3. Antioxidant activity assay

The DPPH radical scavenging activity was measured by the method of Blois et al. (1958). 180 μL of a 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was added to 20 μL of TLWE, diluted to various concentrations. After reacting in the dark for 10 min, the absorbance of DPPH free radicals was measured at 517 nm using a microplate reader (SUNRISE, TECAN Austria GmbH). DPPH radical scavenging activity was calculated from the difference in absorbance of the sample solution, as follows. Ascorbic acid was used as the control.

$$\text{DPPH radical scavenging activity (\%)} = [1 - ((A_{sample} - A_{blank})/ A_{control})] \times 100$$

$ABTS^+$ radical scavenging activity was measured by the method of Re et al. (1999). 7.4 mM ABTS and 2.6 mM potassium persulfate were mixed at a ratio of 1:1 and then left in the dark for 16 h to form a radical. The $ABTS^+$ solution in which the radical was generated was diluted at 734 nm until the absorbance was 0.70±0.02. Twenty microliters of TLWE was added to 180 μL of the diluted $ABTS^+$ solution, left to react for 15 min at room temperature, and the absorbance was measured at 700 nm. Ascorbic acid was used as the control.

$$ABTS^+ \text{ radical scavenging activity (\%)} = [1 - ((A_{sample} - A_{blank})/ A_{control})] \times 100$$

4.4. Cell line and culture

B16F10 mouse melanoma cells were purchased from the Korean Cell Line Bank. The culture medium was 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin, in Dulbecco's modified Eagle's medium (DMEM), and the cells were cultured by passage at 37 °C and 5 % CO_2 at intervals of 4 days.

4.5. Cell viability assay

An MTT assay was performed to confirm the cell viability after TLWE treatment. B16F10 cells were dispensed at 0.8×10^4 cells/well in 24-well plates. After 24 h of pre-culture, the medium was removed and treated with TLWE (50, 100, 200, 400, and 800 μg/mL), diluted in fresh medium. After incubation for 72 h, the medium was removed and the MTT reagent was treated with 0.2 mg/mL. The purple formazan crystals generated were dissolved in DMSO, and absorbance was measured at 570 nm by transferring 200 μL to a 96-well plate. Cell viability was expressed by calculating the percentage absorbance of the untreated control.

4.6. Melanin content assay

A melanin content assay was performed to measure the effect of TLWE on melanin production in cells. B16F10 cells were dispensed at 0.4×10^5 cells/plate on a 60-mm plate and pre-cultured for 24 h. TLWE (50, 100, and 200 μg/mL) was added, and α-MSH was added to 200 nM. α-MSH was used as a negative control, and 200 μM arbutin was used as a positive control. After incubation for 72 h, the medium was removed, and 200 μL of RIPA buffer and inhibitor cocktail (1%) was added and incubated at 4 °C for 20 min to lyse the cells. The lysed cells were scraped using a cell scraper, transferred to a microtube, and centrifuged for 20 min at 21,055 *g* and 4 °C. 1 mL of 10 % DMSO in 1N NaOH was added to the separated pellet and treated at 90 °C for 1 h. A 200 μL sample was transferred to a 96-well plate to measure absorbance at 405 nm, and the results were calculated as a percentage of the α-MSH treatment group.

4.7. Tyrosinase activity assay

B16F10 cells were dispensed at 0.4×10^5 cells/plate on a 60-mm plate and pre-cultured for 24 h. TLWE (50, 100, and 200 μg/mL) was added, and α-MSH was added at a concentration of 200 nM. α-MSH was used as a negative control, and 200 μM arbutin was used as a positive control. After incubation for 72 h, the medium was removed, and 200 μL of RIPA buffer and inhibitor cocktail (1%) was added and incubated at 4 °C for 20 min to lyse the cells. The lysed cells were scraped, using a cell scraper, transferred to a microtube, and centrifuged for 20 min at 21,055 *g* and 4 °C. The protein content of the supernatant was measured using a BCA Protein assay kit and diluted to a protein concentration of 20 μg/μL using 0.1 M sodium phosphate buffer (pH 6.8). Twenty microliters of the diluted protein was added to a 96 well plate, and 80 μL of 2 mg/mL L-DOPA was added. After incubation at 37 °C for 2 h, absorbance was measured at 490 nm, and the results were expressed as a percentage of the α-MSH-treated group.

4.8. Western blot analysis

B16F10 cells were dispensed at 0.4×10^5 cells/plate on a 60-mm plate and pre-cultured for 72 h. TLWE (50, 100, and 200 μg/mL) was added, and α-MSH was added at a concentration of 200 nM and cultured for 24 h. The cells were lysed with 200 μL of

RIPA buffer containing inhibitor cocktail (1%) at 4 °C for 20 min. The lysed cells were scraped, using a cell scraper, transferred to a microtube, and centrifuged for 20 min at 21,055 *g* and 4 °C. The protein content of the supernatant was measured using a BCA Protein assay kit and diluted to a protein concentration of 40 μg/μL. After adding an equal amount of 2×Laemmli sample buffer and treating at 100 °C for 5 min, proteins were separated by loading 10 μL each into an SDS-PAGE gel. After transfer to the PVDF membrane, it was blocked overnight with 5 % skim milk. The membrane was washed 6 times at 10-minute intervals using Tris-buffered saline containing 0.1 % Tween 20 (TBS-T), and then the primary antibody was treated for 2 h. Afterwards, the membrane was washed 6 times with TBS-T and the secondary antibody was added for 2 h. The cells were washed again, several times, with TBST and reacted with ECL solution to develop with Chemidoc (Fusion solo 6S.WL, VILBER LOURMAT, France). The results were compared with the β-actin protein content, and the results were plotted.

4.9. High-performance liquid chromatography (HPLC) analysis

The high-performance liquid chromatography (HPLC) instrument used for the analysis was an Alliance e2695 separations module and 2998 PDA detector (Waters, USA), and the data analysis S/W was analyzed using Waters' Empower system. Standard test curves were prepared by diluting the catechin and *p*-coumaric acid indicators used in HPLC analysis at concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 μg/mL. The methods of Gottumukkala et al. (2014) and Karthikeyan et al. (2015) were modified, for the analysis. The analysis and elution conditions are shown in Table 3.

Table 3: HPLC chromatographic conditions for catechin and *p*-coumaric acid

Control factor	Conditions	
	Catechin	*p*-Coumaric acid
Inject volume	10 μL	
Column	YMC Triart C18 (5 μm, 4.6 x 250 mm)	
Mobile phase	A : 0.01% phosphoric acid B : acetonitrile	Water : MeOH : acetic acid (64 : 34 : 1 v/v)
Flow rate	1 mL/min	
Column temperature	30 °C	25 °C
Wavelength	280 nm	310 nm
Detector	2998 PDA (Waters, USA)	
Separation module	Alliance e2695 (Waters, USA)	

4.10. Human skin primary irritation test

The human skin primary irritation test with TLWE was evaluated by the Korea Dermatology Research Institute. The test was conducted for 3 days from November 20, 2018 (Test No. KDRI-2018-552-Q1, IRB Approval No. KDRI-IRB-18552). TLWE was diluted in squalene to a concentration of 800 μg/mL for use in the test. A total of 30 subjects (5 males and 25 females) participated in the test, and the average age was 27 years. The test site was wiped dry with 70 % ethanol, 15 μL of the sample was applied to a Finn chamber, placed on the back of the subject, and fixed with plaster for 24 h. Subsequently, the patch was removed, and dermatologists observed the test site after 30 min, 48 h, and 72 h. The results were evaluated according to the criteria applied by the International Contact Dermatitis Research Group (ICDRG) and the safety evaluation guidelines of the Personal Care Products Council (PCPC). Using the skin reaction scores of the subjects (Table 4), the irritation index was calculated as follows: After calculating the average stimulation index, the degree of irritation was determined according to (Table 5) to which the Draize Dermal Classification System and EPA (Environmental Protection Agency) Standard Procedure Dermal Classification System were applied.

$$\text{Irritation Index} = \frac{\sum \textit{Irritation score at 24, 48, and 72 hr}}{\textit{Total number of observations}}$$

Table 4: Skin irritation score system

Grade	Description of clinical observation
0(-)	No signs of inflammation, normal skin
0.5(±)	Doubtful or slight reaction
1(+)	Slight erythema
2(++)	Moderate erythema with or without partial edema or papules
3(+++)	Moderate erythema with diffuse edema
4(++++)	Intense erythema with diffuse edema with vesicles

Table 5: Skin irritation judgment

Range of response	Judgement
0 ≤ R < 0.02	No irritancy
0.02 ≤ R < 0.25	Low irritancy
0.25 ≤ R < 01	Slight irritancy
1 ≤ R < 2.5	Moderate irritancy
2.5 ≤ R	Severe irritancy

4.11. Statistical analysis

All experiments were performed three or more times, and data are expressed as mean±standard deviation (SD). Statistical significance and *p*-values were calculated using student's t-test for all experiment results (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Acknowledgements: This research was supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for The Industries of Economic Cooperation Region (P0006063).

Conflicts of interest: None declared.

References

An SM, Koh JS, Boo YC (2010) p- Coumaric acid not only inhibits human tyrosinase activity in vitro but also melanogenesis in cells exposed to UVB. Phytother Res 24: 1175–1180.

An SM, Lee SI, Choi SW, Moon SW, Boo YC (2008) *p*-Coumaric acid, a constituent of Sasa quelpaertensis Nakai, inhibits cellular melanogenesis stimulated by alpha-melanocyte stimulating hormone. Br J Dermatol 159: 292–299.

Bentley NJ, Eisen T, Goding CR (1994) Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol Cell Biol 14: 7996–8006.

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181: 1199.

Choi BK, Lee CN (2015) A study on the synecological values of the Torreya nucifera forest (Natural Monument No. 374) at Pyeongdae-ri in Jeju Island. Korean Institute of Traditional Landscape Architecture 33(4): 87–98.

Chung YC, Ko JH, Kang HK, Kim S, Kang CI, Lee JN, Park SM, Hyun CG (2018) Antimelanogenic effects of *Polygonum tinctorium* flower extract from traditional Jeju fermentation via upregulation of extracellular signal-regulated kinase and protein kinase B activation. Int J Mol Sci 19: 2895.

Floegel A, Kim DO, Chung SJ, Koo SI, Chun OK (2011) Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. J Food Compos Anal 24: 1043–1048.

Friedmann PS, Gilchrest BA, (1987) Ultraviolet radiation directly induces pigment production by cultured human melanocytes. J Cell Physiol 133: 88–94.

Gottumukkala RV, Nadimpalli N, Sukala K, Subbaraju GV (2014) Determination of catechin and epicatechin content in chocolates by high-performance liquid chromatography. Int Scholar Res Notices 2014: 628196.

Jeon HS, Lee YS, Kim NW (2009) The antioxidative activities of Torreya nucifera seed extracts. J Korean Soc Food Sci Nutr 38: 1–8.

Jian D, Su J, Chen W, Hu X, Kuang Y, Xie H, Li J, Chen X (2011) Diethylstilbestrol enhances melanogenesis via cAMP-PKA-mediating up-regulation of tyrosinase and MITF in mouse B16 melanoma cells. Steroids 76: 1297–1304.

Jiménez-Cervantes C, Solano F, Kobayashi T, Urabe K, Hearing VJ, Lozano JA, García-Borrón JC (1994) A new enzymatic function in the melanogenic pathway. The 5,6-dihydroxyindole-2 carboxylic acid oxidase activity of tyrosinase-related protein-1 (TRP1). J Biol Chem 269: 17993–8000.

Kang YG, Choi EJ, Choi Y, Hwang JK (2011) 5,7-Dimethoxyflavone induces melanogenesis in B16F10 melanoma cells through cAMP-dependent signaling. Exp. Dermatol 20: 445–447.

Karthikeyan R, Devadasu C, Srinivasa Babu P (2015) Isolation, characterization, and RP-HPLC estimation of P-coumaric acid from methanolic extract of durva grass (Cynodon dactylon Linn.)(Pers.). Int J Anal Chem 2015: 201386.

Kim DS, Park SH, Kwon SB, YOun SW, Park KC (2004) Effects of lysophosphatidic acid on melanogenesis. Chem Phys Lipids 127: 199–206.

Kim J (2010) Chemical constituents from needles of Torreya nucifera. Planta Med 76: P383.

Kim KN, Yang HM, Kan SM, Kim D, Ahn G, Jeon YJ (2013) Octaphlorethol A isolated from Ishige foliacea inhibits α-MSH-stimulated induced melanogenesis via ERK pathway in B16F10 melanoma cells. Food Chem Toxicol 59: 521–526.

Kim SB, Kim BW, Hyun SK (2018) Comparison of antioxidant activities and effective compounds in Korean and Chinese Torreya seeds. J Korean Soc Food Sci Nutr 50: 274–279.

Kim SH, Park JG, Hong YD, Kim E, Baik KS, Yoon DH, Kim S, Lee MN, Rho HS, Shin SS, Cho JY (2016) Src/Syk/IRAK1-targeted anti-inflammatory action of Torreya nucifera butanol fraction in lipopolysaccharide-activated RAW264.7 cells. J Ethnopharmacol 188: 167–176.

Kim T, Choi HJ, Eom SH, Lee J, Kim TH (2014) Potential alpha-glucosidase inhibitors from thermal transformation of (+)-catechin. Bioorg Med Chem Lett 24: 1621–1624.

Ko GA, Kim Cho SM (2018) Phytol suppresses melanogenesis through proteasomal degradation of MITF via the ROS-ERK signaling pathway. Chem Bio Int 286: 132–140.

Lee A, Kim JY, Heo J, Cho DH, Kim HS, An IS, An S, Bae S (2018) The inhibition of melanogenesis via the PKA and ERK signaling pathways by Chlamydomonas reinhardtii extract in B16F10 melanoma cells and artificial human skin equivalents. J Microbiol Biotechnol 28: 2121-2132.

Lee EH, Kim MU, Kang IK, Park KI, Cho YJ (2018) Inhibitory activities of extracts from Hypericum ascyron L. on biological enzymes. J Korean Soc Food Sci Nutr 47: 7–14.

Lee SI, Kim JS, Jung HW (2017) The antioxidant activities and neuroprotective effects of hot water extracts from Torreyae semen. Korea J Herbol 32: 41–48.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med 26: 1231–1237.

Sato K, Toriyama M (2009) Depigmenting effect of catechins. Molecules 14: 44254432.

Vachtenheim J, Borovanský J (2010) Transcription physiology of pigment formation in melanocytes: central role of MITF. Exp Dermatol 19: 617–627.

Yu L, Haley S, Perret J, Harris M, Wilson J, Qian M (2002) Free radical scavenging properties of wheat extracts. J Agric Food Chem 50: 1619–1624.