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Antimelanogenic effects of *Inula britannica* flower petal extract fermented by *Lactobacillus plantarum* KCCM 11613P*

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Abstract: The inhibitory effects of *Lactobacillus plantarum*-fermented and non-fermented *Inula britannica* extracts on the tyrosinase activity were comparatively investigated to examine whether and how they improve the whitening activity, and the contents of total flavonoids and polyphenolics as bioactive compounds were determined. The skin whitening activity using in vitro or ex vivo tyrosinase and L-3,4-dihydroxyphenylalanine (L-DOPA) staining was examined. The total flavonoid content (TFC) was increased by 13.4% after 72 h-fermentation. The viabilities of the B16F10 cells treated with the fermented and non-fermented control extracts were 100.26% and 92.15% at 500 μg/ml, respectively. In addition, the inhibition of tyrosinase activity was increased by the fermented samples from 29.33% to 41.74% following fermentation for up to 72 h. The tyrosinase activity of the untreated control group was increased to 145.69% in B16F10 cells. The results showed that *I. britannica* fermented by *L. plantarum* dose-dependently inhibited tyrosinase activity, which was stimulated by α-melanocyte stimulating hormone. These results suggest that lactic fermented *I. britannica* extracts can be used as effective skin-whitening materials.

Key words: *Inula britannica*; Flavonoid; *Lactobacillus plantarum*; Antioxidant; Tyrosinase inhibitor
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1 Introduction

Recently, many substantial studies have been focused on developing new antimelanogenic agents using bioactive compounds from natural resources (Smit *et al*., 2009). Especially, Asian women have popularly used several traditional herbs as skin-whitening cosmetics. Additionally, there is a high demand for the development of non-toxic and more effective skin-whitening cosmetics. Although many researchers have attempted to identify pharmacological and cosmetic compounds that inhibit tyrosinase activity and to establish their melanogenic pathways with the goal of whitening skin, very few materials have been developed to date (Gillbro and Olsson, 2011). Traditional herbs are considered as useful materials for the development of skin-whitening cosmetic agents because of their low side effects and environment-friendly impression compared with synthetic chemical compounds.

Melanin is known to be formed through the tyrosine pathway involving auto-oxidation and serves as a light-absorbing compound, which protects the skin against harmful ultraviolet (UV)-light (Gilchrest and Eller, 1999). Melanin synthesis is a complex process and requires numerous factors, including tyrosinase, autocrine and paracrine cytokines, tyrosinase-related protein (TRP)-1, TRP-2, and other enzymes

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for melanin transportation and decomposition (Videira *et al*., 2013). However, the precise inhibitory mechanism underlying the effect of traditional herbs is not yet completely understood.

Natural extracts are commonly used ingredients in numerous cosmetic preparations and homeopathic remedies, and plant extracts with inhibitory effects on melanin synthesis may be a suitable material to be applied for cosmetics due to their relatively low toxicity on human health. Plant extracts containing various phenolic compounds have been discovered to have tyrosinase inhibitory activity, but some of them are highly cytotoxic to melanocytes (Wang K.H. *et al.*, 2006). Therefore, very few compounds are applied currently in the cosmetic industry as industrial skin-whitening ingredients.

Inula britannica has been used as a traditional pharmaceutical plant because of its various effects such as treatment of inflammation, bronchitis, and digestive disorders (Lee *et al.*, 2016). In particular, Bai *et al*. (2005) reported that extract of this flower petal has antibacterial, antihepatitic, antidiabetic, and anticancer effects. In addition, high amounts of biologically active compounds such as sesquiterpene lactones, phenolic acids, and flavonoids are contained in Flos Inulae obtained from *Inula* spp. (Huang *et al.*, 2013).

Fermentation technology has been used to improve food quality, nutritional value, and organoleptic properties (Bourdichon *et al.*, 2012). In this technology, the use of starter cultures is a very promising method industrially for consistent quality and safety of fermented products. This may be attained by inoculating high numbers of viable starter cells, which also effectively destroys contaminated microorganisms during typical food fermentation processes.

Lactobacillus plantarum is one of important starter cultures for lactic fermentation. A distinctive character of fermentation by lactic acid bacteria (LAB) is the production of various organic acids as metabolites of some components in the raw material and the resultant decrease in pH of the culture (Wu *et al*., 2011). In the last few years, bioconversion technology using fungi or bacteria has been used to enhance bio-functional activity in the food industry (Kim *et al*., 2016; Liu *et al*., 2016). LAB have various beneficial effects on human health including their antimicrobial effects. Some possible mechanisms of the action of LAB may be the production of organic acids as well as other by-products of bacterial metabolism in culture (Tsai *et al.*, 2013).

Therefore, the objective of this study is to investigate the tyrosinase inhibitory activity of *I. britannica* extracts fermented by *L. plantarum*, and their potential usefulness for developing skin-whitening agents.

2 Materials and methods

2.1 Plants and bacterial strain

I. britannica was harvested in the Namwon region, Korea, and was obtained from the Herb Kingdom Agriculture Corporation in Namwon, Korea. *I. britannica* plant material was oven-dried using an OF12GW forced convection oven (Jeio-Tech Co., Seoul, Korea) at 60 °C for 10 h until the moisture content was 4%–5% (w/w). Then, the dried *I. britannica* was milled to a particle size of less than 70 mesh with a high-speed mixer (Blender 7012S, Waring, CT, USA) and was stored at 4 °C before use.

L. plantarum KCCM 11613P was previously isolated from a Korean traditional fermented food and maintained at −80 °C in Man, Rogosa, and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) with glycerol (20%, v/v) before use.

2.2 Extraction and microbial fermentation of *I. britannica* and culture conditions

The dried petals of *I. britannica* (20 g, moisture content 9.2% (w/w)) were extracted with 500 ml distilled water for 24 h at 60 °C in a water bath. After extraction, the slurry was filtered through a Whatman No. 2 filter paper (extract I) and extraction of this slurry was performed twice and the resulting extracts were gathered and stored in a deep freeze at −20 °C before use.

The *I. britannica* fermentation medium was prepared by mixing 0.5 g of peptone (0.1%, 1 g/L) in 500 ml of the extract solutions, followed by autoclaving at 121 °C for 15 min and then cooling. Then, *L. plantarum* strain (2% (v/v), initial microbial content approximately 10^6 colony-forming unit (CFU)/ml) or 1 ml of 0.1% peptone water (non-fermented extract) was added into the mixture. After mixing, the samples were cultured at 30 °C with shaking at the rate of 50 r/min for 72 h. During incubation, samples were examined at 0, 4, 8, 12, 24, 48, and 72 h.

2.3 Determination of pH, total titratable acidity, and the growth of cells during fermentation

Each fermented sample (5 g) and 0.1% peptone solution (45 ml) were mixed for the measurement of pH and total titratable acidity (TTA) (Yang *et al.*, 2014). The pH was determined using a pH meter (Model 720, WTW Co., Germany) while the TTA was titrated up to pH 8.2 with 0.1 mol/L NaOH. At each sampling time, the samples were serially diluted with 0.1% sterile peptone solution and the appropriate dilutions were plated onto an MRS agar. The viable cell numbers during fermentation were enumerated by duplicate plating onto MRS agar after incubation, and then the viable cells were counted after 48 h incubation at 37 °C.

2.4 Determination of total polyphenols

The total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent method with slight modifications (Dong *et al.*, 2014). Briefly, 100 μl aliquot of the fermented samples was mixed with 2 ml of 20 g/L Na_2CO_3 and allowed to react for 3 min, then 100 μl 50% Folin-Ciocalteu reagent (Sigma-Aldrich Co., MO, USA) was added and stood to react for 30 min at 25 °C and compared with a gallic acid standard (Sigma-Aldrich Co., MO, USA) under similar conditions. The samples were determined at 750 nm with a spectrophotometer (2120UV, Optizen, Daejon, Korea) and the amount of total polyphenol was expressed as mg gallic acid/g of solid (dry weight, gallic acid equivalent (GAE)).

The total flavonoid content (TFC) was determined by the aluminum chloride ($AlCl_3$) colorimetric method (Lin and Tang, 2007). Briefly, the fermented and non-fermented samples were dissolved in distilled water (1 mg/ml) and a 100-μl aliquot was mixed with 100 μl each of 10% (100 g/L) ammonium nitrate and 1.0 mol/L potassium acetate, and 4.7 ml 80% ethanol. After incubation at 25 °C for 40 min, the absorbance of the reaction mixture was determined at 415 nm. Quercetin (Sigma-Aldrich Co., MO, USA) was used as the standard, and the amount of TFC was expressed as mg quercetin equivalents/g of solids (mg QE/g).

2.5 Cell lines and culture conditions

Mouse melanoma cells (B16F10) were taken from Korean Cell Line Bank (Seoul National University, Seoul, Korea) and routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), streptomycin (100 μg/ml), and penicillin (100 U/ml). The cell lines were cultured at 37 °C under an atmosphere of 5% (v/v) CO_2 with a CO_2 incubator (MCO-18AIC, SANYO Electric Co., Ltd., Japan). For experimentation, adherent cells were detached from the culture dishes using 0.25% (2.5 g/L) trypsin (Life Technologies, Inc., CA, USA).

2.6 Cell viability assay

To determine the safety of the various extracts, the viability of cells treated with the extracts was determined using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. This method is based on the conversion of MTT to MTT-formazan crystals by a mitochondrial enzyme (Wang X. *et al.*, 2006). Briefly, B16F10 cells were inoculated at a density of 5×10^4 cells/well in a 96-well plate and left to adhere overnight. MTT (2.5 ml; Sigma-Aldrich Co., MO, USA) solution and the fermented *I. britannica* extracts (final concentrations 0.05, 0.10, 0.25, and 0.50 mg/well) were prepared in phosphate-buffered saline (PBS), and then the cells were incubated with the extracts for 44 h at 37 °C. After extract incubation, the extract-containing medium was discarded, and then 100 μl MTT solution was added. After incubation for 4 h, the supernatant was removed and 100 μl dimethyl sulfoxide (DMSO) was added to each microwell to dissolve the colored formazan crystals produced by the reaction of cells with MTT. The results were measured at 570 nm using a microplate reader (EL311, Bio-Tek Instrument Inc., Seoul, Korea). The determinations of cell viability were carried out in triplicate under all the treatment conditions, and the cell viability was calculated as $100\%-[1-(A_{sample}/A_{control})]\times100\%$, where A_{sample} and $A_{control}$ are the absorbances of the sample and the control, respectively, after the reaction.

2.7 Mushroom tyrosinase inhibitory activity in vitro

Tyrosinase inhibitory activity was examined using the method of Kubo *et al.* (2003). Briefly, the reaction mixture consisted of 0.1 ml each of the samples and mushroom tyrosinase (1000 U/ml) and 3.8 mmol/L of L-3,4-dihydroxyphenylalanine (L-DOPA;

Sigma-Aldrich Co., MO, USA) in a sodium phosphate buffer (pH 6.8) (total volume of 3.0 ml) and was incubated at 25 °C for 5 min. The absorbance was measured at 475 nm using a spectrophotometer. A blank has all the components except L-DOPA. The tyrosinase inhibitory activity (%) was calculated by $[1-(A_{control}-A_{sample})/A_{control}]\times100\%$, where $A_{control}$ and A_{sample} are the absorbances of the control and the sample, respectively.

2.8 Cellular tyrosinase assay

Tyrosinase activity was determined based on the method described by Lee *et al.* (2006) with slight modifications. Briefly, B16F10 cells were cultured at a density of 5×10^4 cells/well in 6-well plates. After 24 h incubation, α-melanocyte stimulating hormone (α-MSH) (100 μmol/L) was added and cells were treated with fermented and non-fermented *I. britannica* extracts for 3 d. The cells were washed twice with ice-cold PBS and lysed with 0.01 mol/L sodium phosphate buffer (pH 7.0) containing 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and 0.1 mmol/L phenylmethanesulfonyl fluoride (PMSF). After determining the protein content using a Bio-Rad protein assay kit, the lysates were adjusted with lysis buffer to contain equal amounts of protein (40 μg). Then, the lysates were added to the wells of 96-well plates containing 100 mmol/L catechol in 0.1 mol/L phosphate buffer, pH 6.8. After incubation at 37 °C for 1 h, the absorbance was measured at 475 nm using a microplate reader, and the tyrosinase activity of the protein samples was calculated as $A_{475sample}/A_{475control}\times100\%$, where $A_{475control}$ and $A_{475sample}$ are the absorbances at 475 nm of the control and the sample, respectively.

2.9 L-DOPA staining for microscopic observation

L-DOPA staining was performed as reported by Yang *et al.* (2015). B16F10 melanoma cells were washed twice with PBS, fixed for 20 min with 10% formaldehyde solution in PBS, washed three times with PBS, and then incubated with 10 mmol/L L-DOPA at 37 °C for 3 h in the dark. After incubation, the cells were washed twice with PBS. After fixing for 30 min in 10% formaldehyde, the cells had been washed mildly with PBS and the pigmentation was observed using an inverted fluorescence microscope (Olympus IX51 Clone, Olympus Melville, USA).

2.10 Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 18 software (SPSS, Chicago, IL, USA). The mean values were determined using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests and the Student's *t*-test. $P<0.05$ was considered significant.

3 Results

3.1 Fermentation

As shown in Fig. 1, the cells attained the stationary phase after 8 h of culturing under the conditions used in this study. The cell number increased to 8.6 log CFU/ml of culture broth. Additionally, the pH appeared to decrease rapidly to 4.4 during culturing but changed only slightly after 24 h. Furthermore, the total acidity increased to up to 1.5 ml as a consumed volume of 0.1 mol/L NaOH.

During fermentation, the change in the concentration of the bioactive compound is an important factor in evaluating the biofunctional activity of plants. Torino *et al.* (2013) and Dong *et al.* (2014) presented that the change in the polyphenol content depended on the method of fermentation and the microbial strains used. Table 1 shows that the TPC of the fermented samples was not changed significantly ($P>0.05$), but the TFC increased by 13.4% after 72 h fermentation. In contrast, the TFC of the non-fermented control extract was decreased by approximately 3%. Dueñas *et al.* (2005) reported that complex polyphenols could be biotransformed into other simpler and more biologically active compounds by microorganisms. In this study, it was presumed that *L. plantarum* also biochemically transformed the polyphenols into other components.

3.2 Cell viability

Fig. 2 shows the effects of the various concentrations of fermented and non-fermented (control) extracts on the cell viability of B16F10 melanoma cells. At a range of 10–5000 μg/ml, the fermented samples exhibited a lower cytotoxic effect than the control, and the viabilities of the B16F10 cells treated with the fermented and control extracts were 100.26% and 92.15%, respectively, at 500 μg/ml. These results

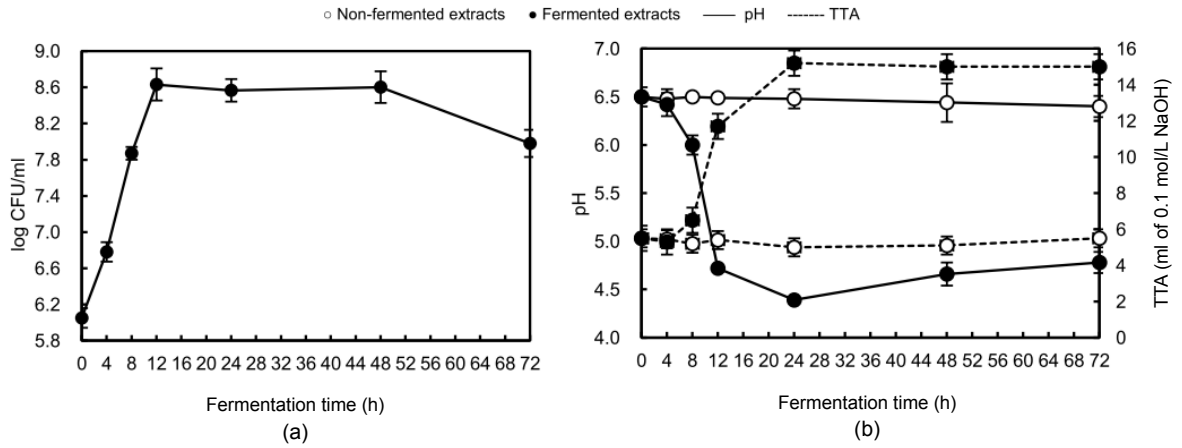

Fig. 1 Determination of pH and titratable acidity of extracts

Growth curves (a), pH and total titratable acidity (TTA) (b) were evaluated during fermentation of *I. britannica* extract by *L. plantarum* at 30 °C for 72 h. Values are expressed as mean±SD

Table 1 Solid, total phenolic and total flavonoid contents of *I. britannica* broth fermented by *L. plantarum*

Fermentation time (h)	Solid content (mg/ml)		Total phenolic content (mg GAE/g of solid)		Total flavonoid content (mg QE/g of solid)	
	NF	IFLP	NF	IFLP	NF	IFLP
0	17.45±0.12	17.10±0.23	6.17±0.33	6.03±0.04	1.95±0.02	1.96±0.02
12		17.80±0.13		6.22±0.19		2.01±0.03
24		17.35±0.10		6.07±0.21		2.02±0.03
48		16.20±0.14		6.25±0.21		2.17±0.03
72		15.80±0.08		6.05±0.26		2.23±0.04

GAE: gallic acid equivalent; QE: quercetin equivalent; NF: non-fermented; IFLP: *I. britannica* fermented by *L. plantarum*. Values are expressed as mean±SD

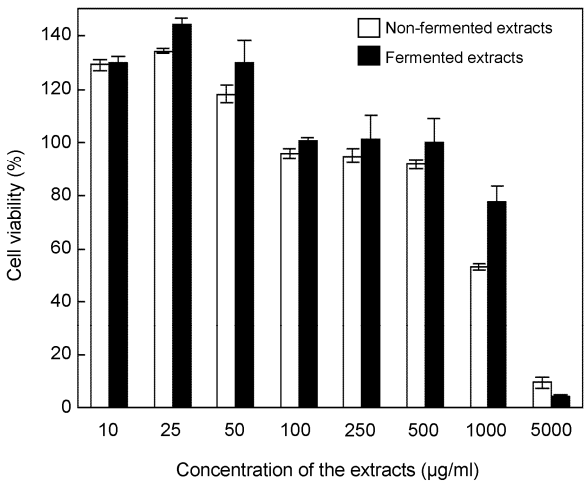

Fig. 2 Cytotoxic effects of *L. plantarum*-fermented *I. britannica* extract on B16F10 melanoma cells

Values are expressed as mean±SD

confirmed that the cell viability was over 90%, and both the fermented and control extracts did not influence the survival of cells at the concentration range used in this study.

3.3 In vitro inhibition of mushroom tyrosinase

Fig. 3 shows the inhibitory effects of *I. britannica* extracts fermented for various times against L-DOPA auto-oxidation using mushroom tyrosinase. The results revealed an apparent increase in the inhibition of tyrosinase activity by the fermented samples from 29.33% to 41.74% following fermentation for up to 72 h. However, the effect of the non-fermented control was not significantly different (P>0.05). Based on these results, all subsequent samples were fermented for 72 h for use in all the experiments in vitro.

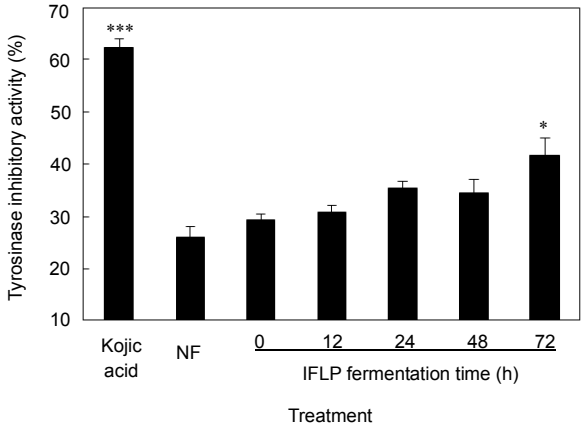

Fig. 3 In vitro tyrosinase inhibitory activity of fermented *I. britannica* water extracts by *L. plantarum* KCCM 11613P

NF: non-fermented extracts; IFLP: *I. britannica* fermented by *L. plantarum*. Kojic acid was used as the positive control to compare antioxidant activities of fermented *I. britannica* extracts. Values are expressed as mean±SD. * $P<0.05$ and *** $P<0.001$ vs. NF

3.4 Inhibition of tyrosinase in B16F10 cells

There are many methods for evaluating whitening effects of novel components on skin. However, Chan *et al.* (2011) reported that the tyrosinase assay using cells was a more credible method than the cell-free mushroom tyrosinase assay to screen skin-whitening compounds. This is mainly because the agents under evaluation target the tyrosinase derived from the melanin-producing cells as opposed to that of mushrooms. α-MSH is known to stimulate the biosynthesis of melanin pigments and, therefore, the production of melanin can be evaluated using this hormone (Más *et al.*, 2003). Hocker *et al.* (2008) presented two possible mechanisms underlying the activation of melanin stimulating signals in melanocytes. First, MSH binds to the melanocortin-1 receptor (MC1R) and activates adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA). The cAMP-activated PKA facilitates the phosphorylation of the cAMP response element binding protein (CREB), which further stimulates the expression of the microphthalmia-associated transcription factor (MITF). Second, Wnt signaling activates the frizzed receptor and regulates β-catenin by glycogen synthase kinase (GSK)-3β. The accumulated β-catenin is then transported to the nuclei where it binds with the T-cell factor (TCF) and lymphoid enhancer factor (LEF). This complex then activates the expression of MITF, which facilitates the synthesis of tyrosinase and tyrosinase-related proteins, such as TRP-1 and TRP-2. Fig. 4 shows the inhibitory effects of the fermented samples on B16F10 melanoma cells following the addition of α-MSH (100 μmol/L) ex vivo, and it appears that the tyrosinase activity of the untreated control group was increased to 145.69%; however, that of the *I. britannica* fermented by *L. plantarum* (IFLP) was decreased gradually to 86.80%. The non-fermented extracts showed no significant difference ($P>0.05$) between samples fermented for different times at a range of 50–500 μg/ml.

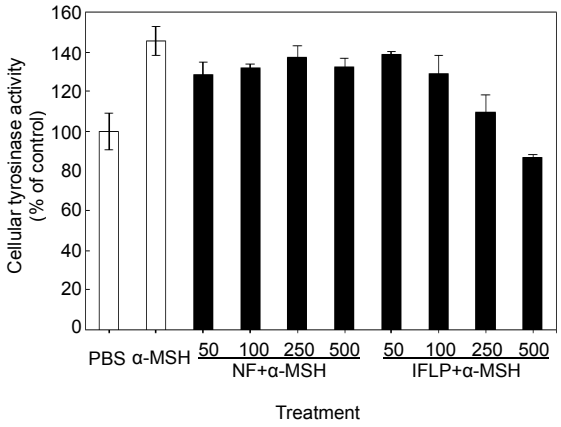

Fig. 4 Comparative effects of *L. plantarum*-fermented and non-fermented *I. britannica* extracts on cellular tyrosinase activity of α-melanocyte stimulating hormone (α-MSH)-stimulated B16F10 melanoma cells

Cells were incubated with 100 μmol/L α-MSH alone or with increasing concentrations of fermented (IFLP) or non-fermented (NF) extracts for 72 h followed by analysis of cellular tyrosinase. Values are expressed as mean±SD

3.5 Determination of tyrosinase activity by L-DOPA staining

L-DOPA staining has been used to evaluate microscopically tyrosinase activity (Goodall *et al.*, 1994). Fig. 5 shows that the control group treated with 100 μmol/L α-MSH had more black-colored spots and dendritic processes than the group treated without α-MSH (only PBS). Furthermore, it appeared that the samples treated with IFLP+α-MSH had fewer black-colored spots and dendritic processes than the

untreated control group (without α-MSH). The results showed that the melanocyte treated with α-MSH exhibited greater tyrosinase activity and had more dendritic processes than the untreated controls (without α-MSH). However, the IFLP inhibited the tyrosinase activity and decreased the number of dendritic processes. Melanocytes, which are known to originate from the neural crest, have dendritic processes and form epidermal melanin units with keratinocytes. The connection of the dendritic processes with the adjacent keratinocytes has a role in the transportation of melanosome because melanosome can be moved to the keratinocyte by the dendritic processes. Furthermore, the production of epidermal melanin can be facilitated by UV light and α-MSH (Chakraborty *et al.*, 1996; Provance *et al.*, 1996).

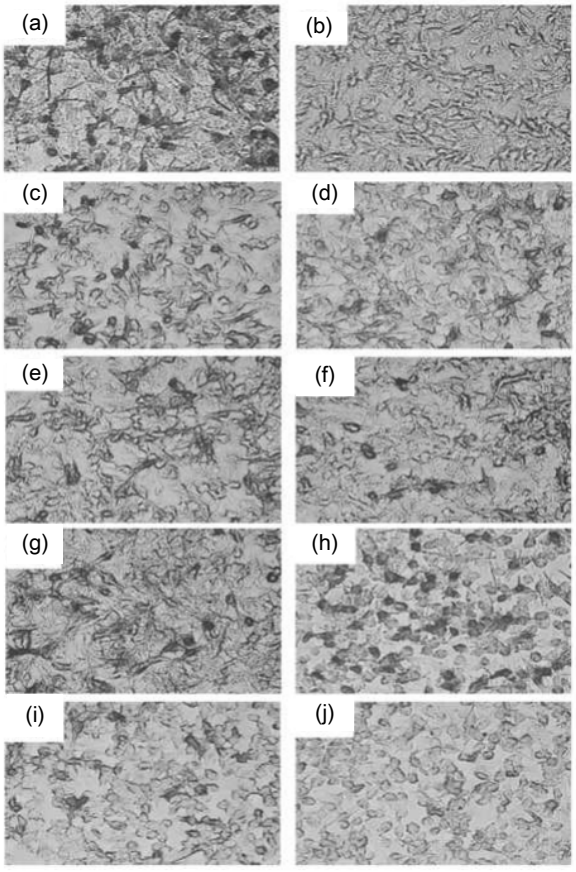

Fig. 5 Determination of tyrosinase activity of α-MSH-induced cells using L-DOPA staining

Cells incubated with α-MSH 100 μmol/L (a), PBS (b), non-fermented extracts of 50 (c), 100 (d), 250 (e), and 500 μg/ml (f), and fermented extracts of 50 (g), 100 (h), 250 (i), and 500 μg/ml (j), respectively, for 72 h

4 Discussion

Khan *et al.* (2010) isolated the secondary metabolites in *I. Britannica*, which found that the major flavonoids in flower petals were patuletin, axillarin, and nepitin and detected a small amount of quercetin and kaempferol. Flavonoids have been known to have various bioactive functions such as therapeutic detoxification against liver injury (Zhang *et al*., 2014) and apoptosis of leukemia cells (Wang *et al*., 2015). In addition, many studies have reported that some phenolic compounds including flavonoids, such as kaempferol, parulerin, luteolin and epigallocatechin, have inhibitory effects on tyrosinase (Kim and Uyama, 2005; Huang *et al.*, 2013). Furthermore, many have studied a fermentation technique of bioactive flavonoids to improve their bioactivity (Xing *et al*., 2016). This study showed that the extracts fermented by *L. plantarum* had greater antioxidant and tyrosinase inhibitory effects than the non-fermented extract, suggesting that the fermentation of *Inula* extracts can be applied to the industrial development of both functional foods and cosmetics.

From the results in this study, it appears that the inhibitory activity of the fermented extract of *Inula* petals on tyrosinase was greater than that of a non-fermented control though the TPC was not changed significantly. It is assumed that some compounds were affected to increase their bioactivity. During fermentation, some bioactive compounds can be bio-transformed and, therefore, our further research studies will focus on determining the biologically active compounds in this extract as well as the mechanisms underlying their bioconversion during fermentation.

Compliance with ethics guidelines

Eun-hye PARK, Won-young BAE, Jae-yeon KIM, Kee-tae KIM, and Hyun-dong PAIK declare that they have no conflicts of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：植物乳杆菌 KCCM 11613P 发酵的旋覆花花瓣提取物的抗黑素生成的作用

目　的：研究植物乳杆菌发酵对旋覆花花瓣提取物对酪氨酸酶的抑制效果，为开发旋覆花提取物作为潜在的皮肤增白成分提供依据。

创新点：发现旋覆花植物乳杆菌发酵的提取物可以作为有效的美白材料。

方　法：比较研究了植物乳杆菌发酵的旋覆花花瓣提取物对酪氨酸酶活性的抑制作用，并测定了提取物中总黄酮和多酚类化合物的含量。通过体外酪氨酸酶和左旋多巴（L-DOPA）染色来测定其对皮肤的美白活性。

结　论：旋覆花花瓣经过 72 小时的发酵后，其提取物中总黄酮含量提高 13.4%。采用 500 μg/ml 发酵和未发酵的提取物处理 B16F10 细胞，细胞活性分别为 100.26%和 92.15%。此外，发酵 72 小时后，该提取物对酪氨酸酶活性的抑制率由 29.33%上升到 41.74%。未处理的对照组 B16F10 细胞酪氨酸酶活性增加到 145.69%。结果表明，旋覆花发酵提取物对酪氨酸酶活性呈剂量依赖性抑制是促黑激素（α-MSH）的刺激引起。旋覆花植物乳杆菌发酵的提取物可以作为有效的美白材料。

关键词：旋覆花；类黄酮；植物乳杆菌；抗氧化剂；酪氨酸酶抑制剂