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Original Research Article

The Suppressive Effect of *Pueraria lobata* Root Extract and Its Biotransformed Preparation against Skin Wrinkle Formation

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Abstract - EP was obtained through 20% ethanol extraction of *Pueraria lobata* root, and the fermented form of EP, FEP, was prepared from the EP after incubating with *Lactobacillus rhamnosus* vitaP1. There was no significant toxicity by EP and FEP up to 1000 ㎍/㎖ in NIH-3T3, HaCaT, and B16F10 cells. In addition to antioxidant potentials of EP and FEP determined by DPPH and ABST assays, we confirmed increase of procollagen type I and elastin synthesis by supplementation of the EP and FEP at the concentration of 50 ㎍/㎖ using ELISA kits. The protein expression levels of matrix metalloprotease (MMP)-1, -3, and -9, those are involved in the degradation of collagen or other skin matrix proteins, were remarkably suppressed while their inhibitory protein metallopeptidase inhibitor 1 (TIMP-1) was greatly up-regulated by supplementation of the EP and FEP at a concentration of 50 ㎍/㎖. Taken together, both EP and FEP supplementation could be involved in the suppression of the skin wrinkle formation through inhibiting degradation of collagen and stimulating the synthesis of collagen and elastin. The results showed that the anti-wrinkle potential of the EP and FEP will be a promising candidate for developing cosmeceutical compounds or products.

Key words - Antioxidant, Fermentation, *Pueraria lobate*, Wrinkle

Introduction

Skin aging involves the structural and physiological alterations and progressive changes in each skin layer as well as changes in skin appearance. Skin aging is a complex biological process influenced by both intrinsic (genetics, cellular metabolism, hormone and metabolic processes) and extrinsic (chronic light exposure, pollution, ionizing radiation, chemicals, toxins) factors (Baumann, 2007; Ganceviciene *et al*., 2012; Lephart, 2016). Skin health and beauty is considered one of the principal factors representing overall "well-being" and the perception of "health" in humans (Ganceviciene *et al*., 2012). Sometimes, whitening agents such as corticosteroids, tretinoin (all-trans retinoic acid) and hydroquinone those medically applied for effectively lighten the skin tone of hyperpigmented lesions, may cause a variety of side-effect (Desmedt *et al*., 2016). Thus an increasing interest in herbs, many studies focused on discovering novel natural skin-whitening agents that are currently underway (Smit *et al*., 2009). Wrinkle formation involves degradation of the skin's three primary structural constituents, collagen, elastin and glycosaminoglycans (e.g., hyaluronic acid) and these three components are known to decline with age or exposure to ultraviolet light). Thus, prevention of wrinkle development has assumed a fundamental status in anti-ageing skin care.

Isoflavones are a subclass of flavonoids, which include over 6000 identified family members and is a phenolic

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compound is rich in soy beans, soy foods, legumes and fungus (Woo *et al*., 2003; Yu *et al*., 2016). Recently, it has been found that root part of *Pueraria thunbergiana* possesses abundant flavonoid (Shin *et al.*, 2000; Han *et al*., 2015). Besides their antioxidant capacities, antimicrobial, and anti-inflammatory activities, isoflavones possess a weak estrogenic (agonistic) activity through its binding to estrogen receptor (Yu *et al*., 2016). Recent efforts are aimed at isoflavone biotransformation to increase the health benefits of isoflavones like as an equol (Gao *et al*., 2016). Moreover, in the topical application of isoflavones on skin whitening, transformation of isoflavones like metabolism by microbiota in the intestine will be necessary since isoflavone itself has less potential bioavailability. In this experiment, we prepared the ethanol extract of the root part of *Pueraria lobata* (EP) and fermented extract (FEP) after biotransformation of the EP with *Lactobacillus rhamnosus* vitaP1. Then, we investigated the anti-wrinkle effect of the EP and FEP, fermented form of EP, based on the production of procollagen type I, elastin, and the suppression of matrix metalloprotease (MMP)-1 (collagenase-1), -3 (stromelysin-1), and -9 (gelatinase). In addition, antioxidant capacity of EP and FEP were determined by DPPH and ABTS method.

Materials and Methods

Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), β-Phycoerythrin, mushroom tyrosinase, and α-melanocyte stimulating hormone (α-MSH) were purchased from Sigma (St Louis, MO, USA). Unless indicated otherwise, all other chemicals were obtained from Sigma.

Preparation of EPL and FEPL

Pueraria lobata (Wild.) Ohwi roots were purchased from Daegu oriental medicine market and identified by Dr. T.H. Park of Daegu University (Daegu, Korea). Roots of *P. lobata* were chopped into small pieces and extracted three times in 20% ethanol for 24 hours each at room temperature (yield 20.5%). The extract was subsequently filtered to remove any particulates and was concentrated under vacuum at 50°C. Then, the concentrated crude 20% EtOH extract of *P. lobata* (EP) was lyophilized to obtain a powder and stored at −20°C for further experiments (20.5% yield). FEP was prepared after fermentation of 2% PE by incubation with *L. rhamnosus* vitaP1 for 48 hours at 37°C with shaking (250 rpm). Then fermented of EP (FEP) was re-extracted with ethyl acetate hexane (EtOAc). EP and FEP were dissolved in phosphate buffer saline (PBS) (Gibco, USA).

Cell culture

NIH-3T3 (mouse embryo fibroblast cell), HaCaT (immortalized human keratinocyte cell), and B16F10 (mouse melanoma cell) were obtained from Korean cell line bank (Seoul, Korea) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 1% penicillin (Gibco, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell cytotoxicity

Cell cytotoxicity was measured by quantitative colorimetric assay with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which measures the mitochondrial activity of living cells. Exponentially growing NIH-3T3, HaCaT, and B16F10 cells were seeded at 1×10^4 cells/well in 96-well tissue culture plates and treated with different dose of EP and FEP (1, 10, 100, 500, 1000 ㎍/㎖) for 24 hours. After incubation of MTT (150 ㎍/㎖) for 4 hours, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and then measured the optical density at 540 ㎚ using a Molecular Devices microplate reader (Menlo Park, CA). Cytotoxicity was expressed as a percent of untreated control cells.

DPPH scavenging activity

Different concentrations of EP and FEP were adjusted at 100 ㎕ with reaction mixture and then reacted with l00 ㎕ of 0.4 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in 99% EtOH. After vigorous shaking, reaction mixtures were allowed to reach a steady state at room temperature for 30 minutes. Decolorization of DPPH was determined by measuring the absorbance at 540 ㎚ with using a Molecular Devices microplate reader.

ABTS assay

The 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) antioxidant assay was generated by adding 7.4 mM ABTS to 2.4 mM potassium persulfate and the mixture was left at room temperature for overnight in dark. The ABTS radical cation solution was diluted with distilled water to obtain an absorbance of approximately 1.1 ± 0.02 (1.09) at 734 ㎚. 400 ㎕ EP and FEP extract at various concentrations was added to 800 ㎕ of ABTS reagent. Reaction was conducted at room temperature for 10 minutes in dark, and then absorbance was measured at 734 ㎚ with using UV/VIS spectrophotometer (Shimadzu, Japan).

Effect of EP and FEP on the production of procollagen type I and elastin

NIH-3T3 cells were inoculated into 24-well plate (1×10^5 cells/well) and were cultured for 24 hours. The media were switched into serum-free DMEM in the presence or absence of EP or FEP (50 ㎍/㎖) for 24 hours. After collecting supernatants, procollagen type I and elastin contents were determined by a procollagen type I C-peptide assay kit (Takara Bio, Japan) and FASTINTM assay (Biocolor, Belfast, Ireland), respectively.

Effect of EP and FEP on the secretion of metallopeptidase inhibitor 1, matrix metalloprotease (MMP)-1, -3, and -9

To determine the secreted forms metallopeptidase inhibitor-1 (TIMP-1), matrix metalloprotease (MMP)-1, -3, and -9 in NIH-3T3 cells were switched into serum free-medium and then EP and FEP (50 ㎍/㎖) were treated for 24 hours. After collecting the culture medium, TIMP-1, MMP-1, -3, -9 levels were measured by each ELISA assay kits (Amesham Pharmacia biotech, Centenial Ave., USA) according to manufacturer's protocol. Culture supernatants were four-fold diluted with assay buffer solution. Equal volume (100 ㎕) of diluted culture medium and peroxidase conjugate (100 ㎕) were mixed and then incubated for 2 hours at room temperature. Then 100 ㎕ of TIMP-1 was added and reacted for 30 minutes. The absorbance of each samples were measured at 450 nm using a Molecular Devices microplate reader.

Statistical Analyses

Each experiment was repeated three or four times, and the results of a representative experiment are shown. The results are expressed as means ± SEM and were analyzed using one-Way ANOVA followed by Turkey's method (Systat Software Inc., San Jose, CA, USA). A statistical probability of $p < 0.05$ was considered significant.

Results

Cell cytotoxicity of EPL and FEPL

To exclude the possible cell cytotoxicity of the EP and FEP, MTT reduction assay was conducted in HaCaT, B16F10, and NIH-3T3 cells. Both EP and FEP up to 1000 ㎍/㎖ did not show any significant cell cytotoxicity on keratinocytes (Fig. 1A), melanocytes (Fig. 1B), and embryonic fibroblast cells (Fig. 1C).

Antioxidant potential of EP and FEP

Antioxidant capacities of the EP and FEP were determined by DPPH (Fig. 2A) and ABTS-based (Fig. 2B) assays. When determined the antioxidant capacities of the EP and FEP based on their free radical scavenging activity, EP showed remarkable antioxidant capacity but in the same amount of FEP the antioxidant capacity was less than that of EP (Fig. 2A). ABTS scavenging activity represents the compound's cationic radical scavenging potential. As shown in Fig. 2B, ABST scavenging activity of the EP and FEP showed the similar results as shown in DPPH assay. These results suggest that both EP and FEP have an antioxidant potential, which has been helpful in reducing oxidative stress involved in skin aging.

Effect of EP and FEP on the production (or secretion) of procollagen type I and Elastin

Collagen and elastin are important proteins involved in forming skin matrix. Reduction in collagen and elastin may be associated with a wrinkle formation due to destruction of skin architecture (Contet-Audonneau *et al*., 1999).

Procollagen type I is cleaved at its carboxy-terminus to produce mature collagen type I, so the content of procollagen type I is a good indicator of collagen biosynthesis (Díez *et al*.,

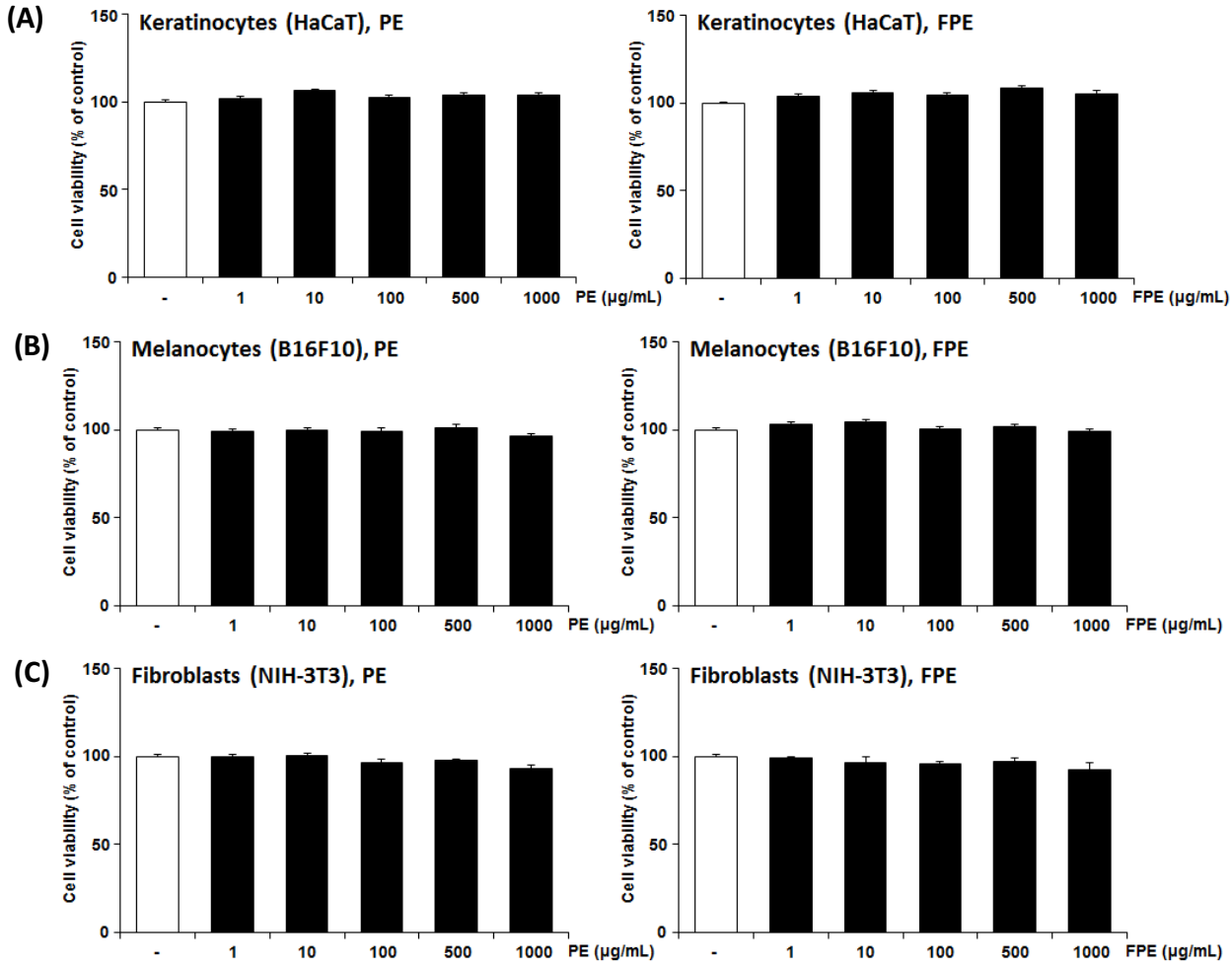

Fig. 1. Cytotoxic effects of PE (*Pueraria lobata* extracts) and Fermented PE (FPE) in (A) keratniocytes (HaCaT), (B) melanocytes (B16F10), and (C) fibroblast cells (NIH-3T3). Cells were treated with indicated concentrations of extracts. Cell viability was measured using the MTT assay. The viability of untreated control cells was defined as 100%. (a) Cells (2×10^4 cells/well) treated with PE and FPE (1 ㎍/㎖ to 1000 ㎍/㎖) for 24 hrs. The results are mean ± S.E. of quintuplicates from a representative experiment (*$p<0.05$; significantly different from the control).

1996). As shown in Fig. 3A, EP (50 ㎍/㎖) and FEP (50 ㎍/㎖) increased the production of procollagen type I by 2.2 fold and 2.4 fold. Test with equol used as a positive control showed 2.2 fold increase of procollagen type I synthesis at same concentration (Fig. 3A). Stimulation of elastin in skin cells will be a good contributor involved in reduction of wrinkle formation. EP and FEP could increase the production of elastin in NIH-3T3 cells by 1.9 and 2.1 fold, compared to untreated control (Fig. 3B). FEP was more potent than that of equol which showed 1.9 fold increase of elastin synthesis.

Effect of EP and FEP on the production (or secretion) of MMP-1, -2, and -3

As a causal promotion of wrinkle formation, it has been suggested that suppression of expression or activities of matrix metalloproteases (MMPs) will be another approach to alleviate wrinkle formation (Pittayapruek *et al*., 2016). When determined the inhibitory effect of EP and FEP on the expression of MMP-1 (collagenase-1), -3 and -9 by ELISA assay (Fig. 4), the supplementation of EP could remarkably suppress the expression level of MMP-1, -3 and -9. In addition, FEP showed more potent activity than that of EP but statistical significance was not observed.

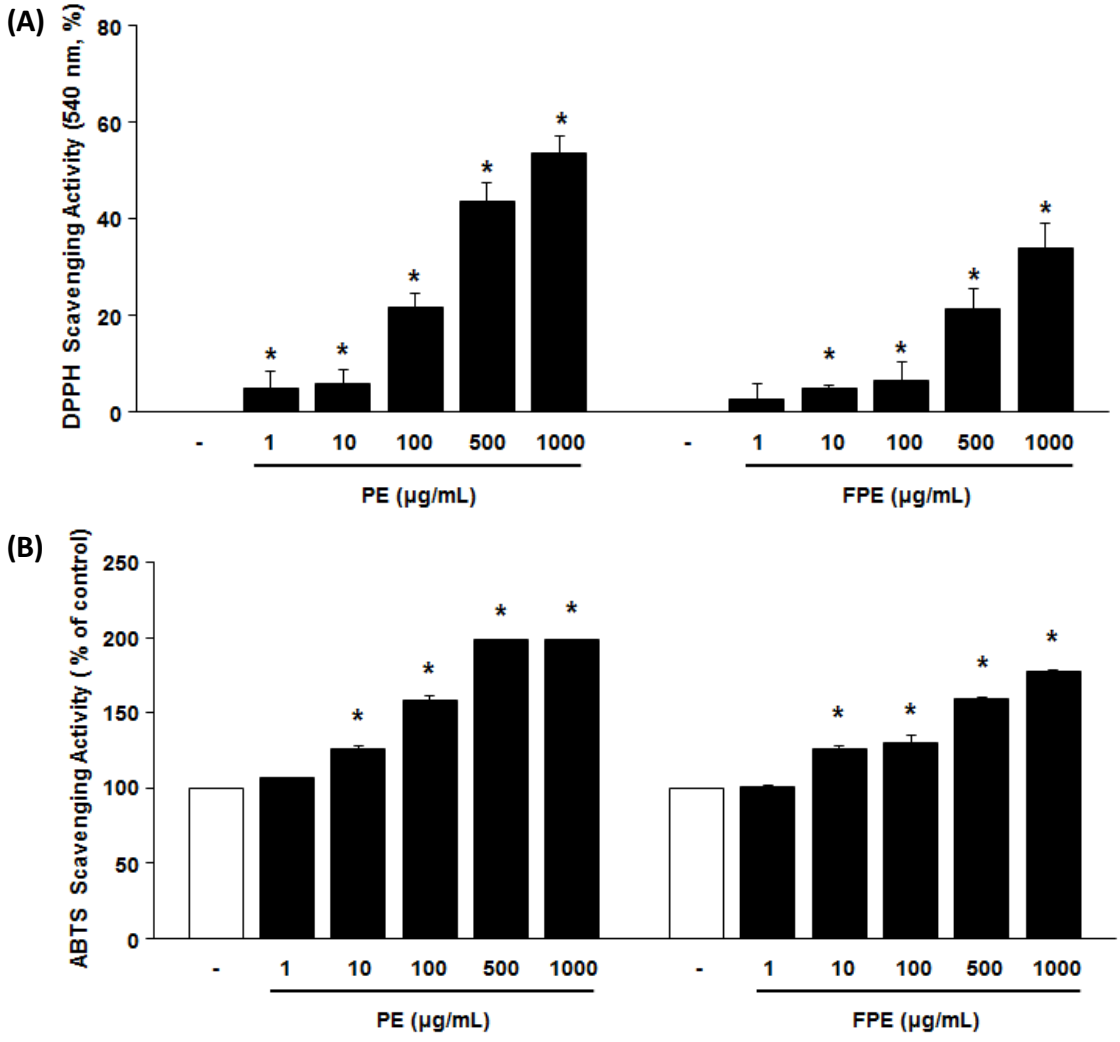

Fig. 2. The antioxidant activity of PE (*Pueraria lobata* extracts) and Fermented PE (FPE) using (A) DPPH radical scavenging activity assay and (B) ABTS radical scavenging activity assay. Each bar indicates the mean S.D. of determinations, n=4. Statistical analysis was performed by the one-way ANOVA ($p<0.05$).

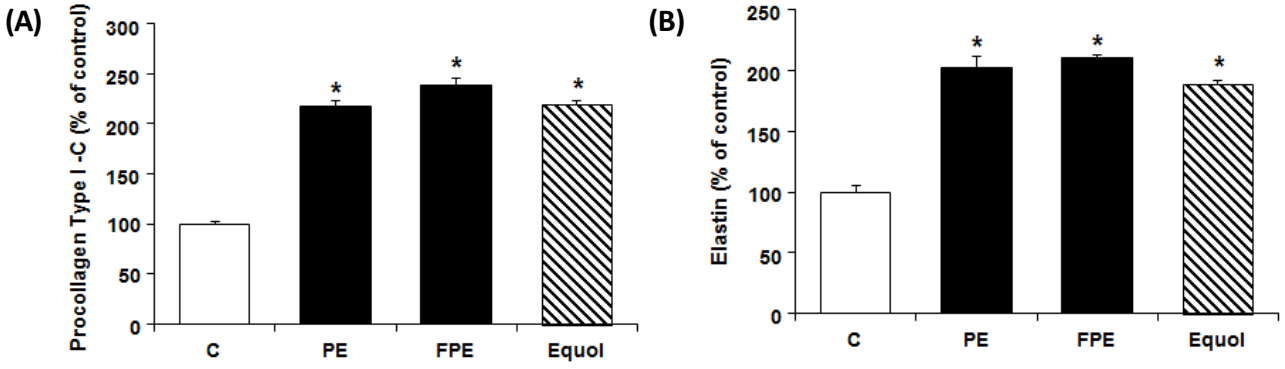

Fig. 3. Effects of PE (*Pueraria lobata* extracts) and Fermented PE (FPE) on the production of (A) type I procollagen and (B) elastin levels by ELISA assay in fibroblast NIH-3T3 cells. The results are mean ± S.E. of quintuplicates from a representative experiment (*$p<0.05$; significantly different from the control).

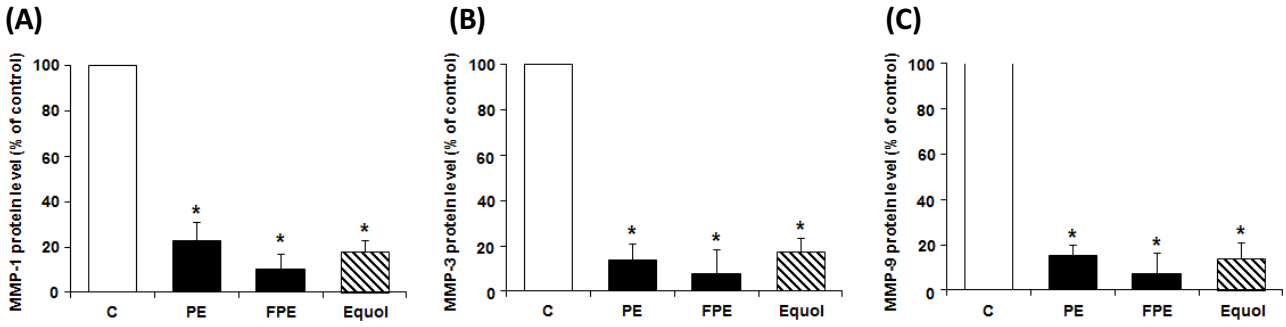

Fig. 4. Inhibitory effects of PE (*Pueraria lobata* extracts) and Fermented PE (FPE) on the expression of (A) MMP-1, (B) MMP-3, and (C) MMP-9 secretion by NIH-3T3 cells. The levels of MMPs were measured by ELISA assay. Data are presented as mean ± S.E. of quintuplicates from a representative experiment (*$p<0.05$; significantly different from the control).

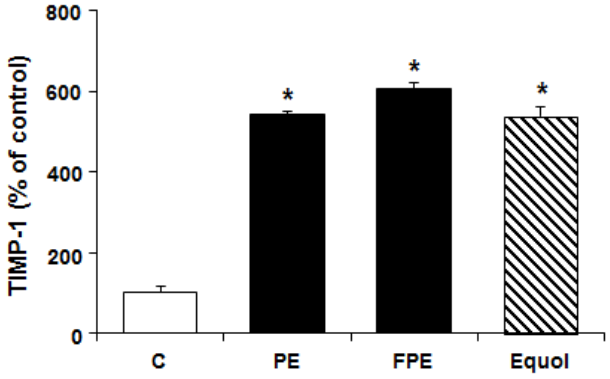

Fig. 5. Effects of PE (*Pueraria lobata* extracts) and Fermented PE (FPE) on the expression of TIMP-1 in NIH-3T3 cells. The level of TIMP-1 was measured by ELISA method. Data are presented as mean ± S.E. of quintuplicates from a representative experiment (*$p<0.05$; significantly different from the control).

Effect of EP and FEP on the production of TIMP-1

In addition to down regulation in protein expression of MMPs, the strategy involved in inhibition of their activities will be another choice in suppression of wrinkle formation. TIMP-1 is an endogenous MMPs inhibitor and behaves as a growth factor-like molecule in keratinocytes and fibroblasts (Hornebeck, 2003). When the effect of EP and FEP on the protein expression of TIMP-1 was determined by ELISA assay, supplementation of EP and FEP could significantly increase the protein expression level of TIMP-1, by 5.4 and 6.5 times more compared to untreated control (Fig. 5). These results suggest that the EP and FEP could not only suppress the protein expression levels of MMP-1, -3, -9 (Fig. 4) but also inhibit the activities of MMP-1, -3, and -9 through upregulation of TIMP-1.

Discussion

In this report, we found that EP supplementation could involve in alleviation of wrinkle formation through suppression of protein expression level of MMP-1, -3, and -9 in conjunction with up-regulation of TIMP-1. In addition, augmentation of protein expression level of procollagen type I and elastin by EP supplementation will be another beneficial effect against wrinkle formation.

Pueraria lobata is a species of climbing plant belonging to the *Leguminosae* family and contains various phenolic compounds and isoflavonoids, including daidzin, daidzein, genistin, and puerarin, used for alleviating problems with liver damage, bone loss (Kim *et al*., 2015), obesity (Lee *et al*., 2015; Jung *et al*., 2017), allergy (Jeong *et al.,* 2013), inflammation (Choo *et al*., 2002) and metabolic disorders such as obesity (Jung *et al*., 2017). The extract of *Pueraria lobata* (EP) has been used as an alternative herb for alleviating postmenopausal symptoms (Woo *et al*., 2003). Many studies have been conducted to find a natural material that has high biologic functions for human skin without any side effects (Smit *et al*., 2009; Lee *et al*., 2012; Kim *et al*., 2015; Chen *et al*., 2016). Recently, it has been found that anti-melanogenic effects of aerial part of *Pueraria thunbergiana in vitro* and *in vivo* (Han *et al*., 2015). In addition to intervention regarding anti-melanogenesis, prevention of wrinkle formation is another concern in skin-aging and beauty. It is noteworthy that looking better is one of important factors in well-being

and quality life. In addition, maintenance of well performing skin barrier is also important point for keeping a good health, since destruction of skin barrier will cause vulnerability to infection, or penetration of environmental pollutants (Ganceviciene *et al*., 2012). Wrinkle formation is one of cutaneous signs of aging due to alterations of collagen structure, composition, function, synthesis, and degradation (Ramos-e-Silva and Carneiro, 2001).

Therefore, the blockage of collagen-degrading MMPs or stimulation of collagen synthesis, or both, can be a good target. In this view, the good starting point would be EP and FEP from natural products. Like other extracts isolated form plants, EP contains antioxidant potential that was confirmed by DPPH (Fig. 2A) and ABST (Fig. 2B) assay. However, it is unclear why FEP was less potent than that of EP even after fermentation process. Interestingly, EP and FEP could suppress the wrinkle formation via increase of collagen and elastin and suppression of MMP-1, -3, and -9 protein levels (Fig. 4). In addition, up-regulation of TMIP-1 by supplementation of EP and FEP further suppressed activities of MMPs (Fig. 5). This result is valuable since more active isoflavone metabolites could be obtained from relatively small starting quantity. It is speculated that *L. rhamnosus* vitaP1 could biotransform from isoflavones of EP into more active forms of isoflavones including dihydrodaidzein (DHD), dihydrogenistein (DHG) or equol, which have more potent estrogenic activity, biological effect and bioavailability.

Besides the inhibitory effect of EP and FEP on the collagen degrading process by MMPs, additional protective effects of the EP and FEP were shown in the view of increase production in collagen (Fig. 3A) and elastin (Fig. 3B), those involve the healthy skin architecture and are diminished with aging or exposure to sunlight (Lephart, 2016; Makrantonaki *et al*., 2016). However, the elucidation of the underlying molecular mechanism involved in down-regulation of MMP-1, -3, and -9 and up-regulation of procollagen type I, elastin and TIMP-1 expression should be under more extensive studies. Especially, the mechanistic insight of robustly increased expression of TIMP-1 by the supplementation of EP and FEP should be paid more attentions.

Taken together, supplementation of EP and FEP could involve in the suppression of parameters associated with wrinkle formation through suppression of MMP-1, -3, and -9 expression and up-regulation of TIMP-1. In addition, acceleration of collagen and elastin synthesis by EP and FEP supplementation may prove to have potential values as anti-wrinkle cosmetic additives.

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