Pueraria lobata root extract and its biotransformed preparation could suppress skin wrinkle formation

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ABSTRACT

The present study investigated that the effects of 20% ethanol extraction of *Pueraria lobata* root (EPL) and its fermented form incubated with *Lactobacillus rhamnosus* vita P1 (FEPL) on anti-oxidative and preventive effects of wrinkle formation. There was no significant toxicity by EPL and FEPL up to 1000 μg/ml in HaCaT, B16F10, and Hs68 cells. In addition to anti-oxidant potentials determined by DPPH and ABST assay, EPL and FEPL at concentration of 50 μg/ml could stimulate the synthesis of procollagen type I and elastin determined by ELISA assay. The protein expression levels of matrix metalloprotease (MMP)-1, -3, and -9, those are involved in degradation of skin matrix proteins, were remarkable suppressed but their inhibitory protein metallopeptidase inhibitor 1 (TIMP-1) was greatly up-regulated by EPL and FEPL. Taken together, both EPL and FEPL supplementation could be involved in suppression of skin wrinkle formation through inhibiting degradation of collagen and stimulating synthesis of collagen and elastin. Given anti-wrinkle potential of EPL and FEPL will be a promising candidate for developing cosmeceutical compounds or products. This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01128102)" Rural Development Administration, Republic of Korea.

Material & Methods

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℃. Then, the concentrated crude 20% EtOH extract of *P. lobata* (EPL) was lyophilized to obtain a powder and stored at –20 ℃ for further experiments (20.5% yield). FEPL was prepared after fermentation of 2% LPE by incubation with *L.rhamnosus* vitaP1 for 48 hours at 37℃ with shaking (250 rpm). Then fermented of EPL (FEPL) was re-extracted with ethyl acetate hexane (EtOAc). EPL and FEPL were dissolved in Phosphate Buffer Saline (PBS) (Gibco, USA).

Cell culture

NIH-3T3 (mouse embryo fibroblast cell), HaCaT (immortalized human keratinocyte cell) , B16F10 (mouse melanoma cell) and Hs68 (human foreskin fibroblast 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 ℃.

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, B16F10 and Hs68 cells were seeded at $1x10^4$ cells/well in 96-well tissue culture plates and treated with different dose of EPL and FEPL (1, 10, 100, 500, 1000 μg/㎖) for 24 hours. After incubation of MTT (150 μg/㎖) for 4 hours, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and then measured the optical density at 540nm 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 EPL and FEPL 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 540nm 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 nm. 400 ㎕ EPL and FEPL extract of 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 EPL and FEPL 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 EPL or FEPL (50 μg/㎖) for 24 hours. After collecting supernatants, pro-collagen 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 EPL and FEPL on the secretion of metallopeptidase inhibitor 1, matrix metalloprotease (MMP)-1, -3, and -9

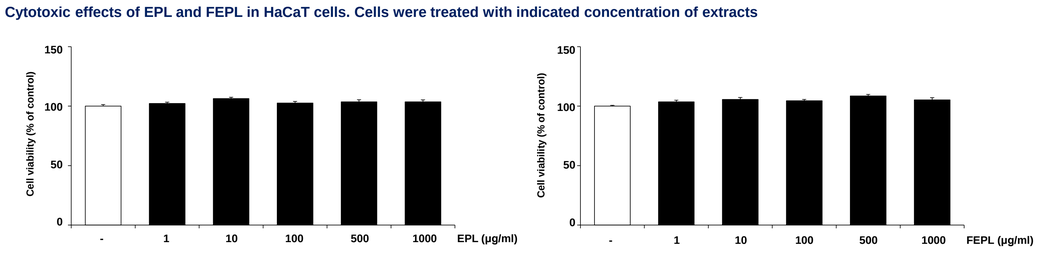
To determine the secreted forms metallopeptidase inhibitor 1 (TIMP-1), matrix metalloprotease (MMP)-1, -3, and -9, expotentially growing NIH-3T3 cells were switched into serum free-medium and then EPL and FEPL (50 μg/㎖) were treated for 24 hours. After collecting the culture medium, TIMP-1, MMP-1, -3, -9 level were measured by each ELISA assay kits (Amesham Pharmacia biotech, Centenial Ave., USA) according to manufacture'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 TMB was added and reacted form 30 minutes. The absorbance of each samples were measured at 450 nm using a Molecular Devices microplate reader.

Statistical Analyses

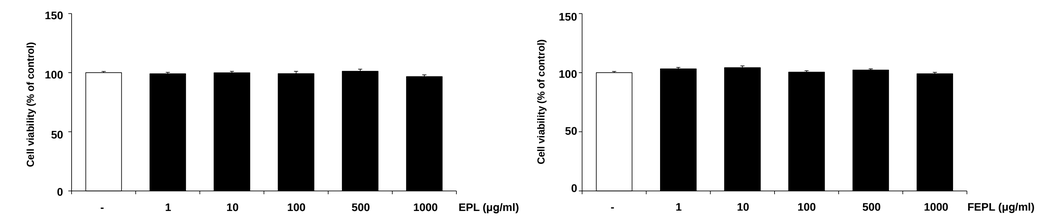
Each experiment was repeated three or four times, and the results of a representative experiment are shown. The results are expressed as means ± SEM and were analyzed using one-Way ANOVA followed by Turkey's method (Systat Software Inc., San Jose, CA, USA). A statistical probability of $p < 0.05$ was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

Result

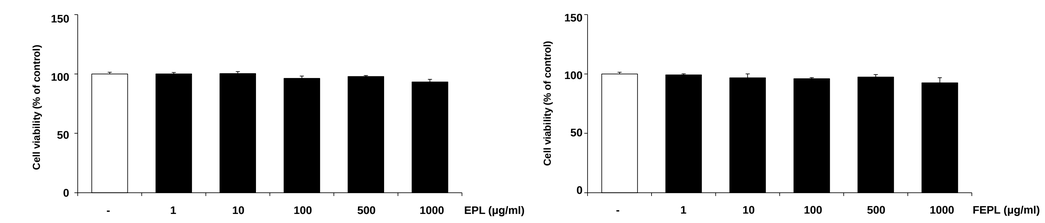
Cytotoxic effects of EPL and FEPL in HaCaT cells. Cells were treated with indicated concentration of extracts



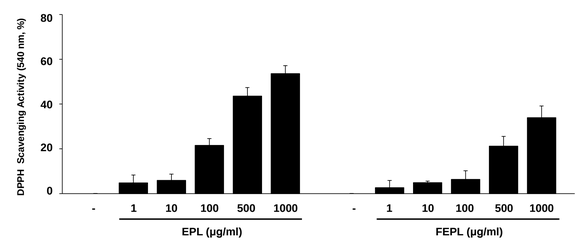
Cytotoxic effects of EPL and FEPL in B16F10 cells. Cells were treated with indicated concentration of extracts



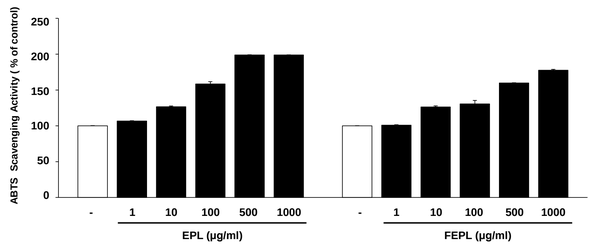
Cytotoxic effects of EPL and FEPL in Hs68 cells. Cells were treated with indicated concentration of extracts



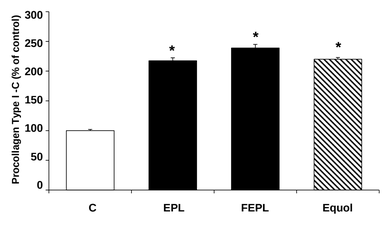
DPPH radical scavenging activity of EPL and FEPL



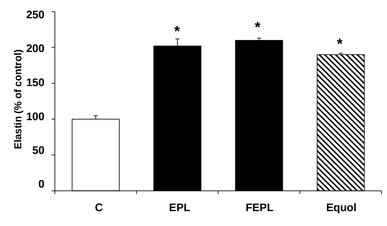
ABTS radical scavenging activity of EPL and FEPL



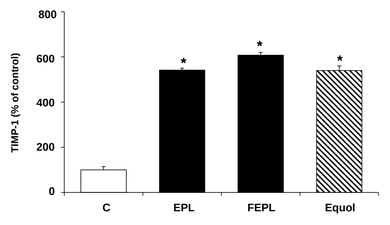
Effect of EPL, FEPL and Equol (50 μg/㎖) on pro-collagen type I synthesis



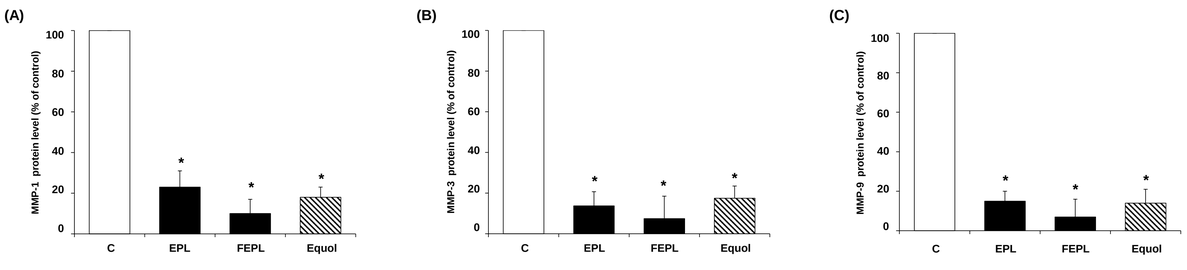
Effect of EPL, FEPL and Equol (50 μg/㎖) on Elastin synthesis



Effect of EPL, FEPL and Equol (50 μg/㎖) on TIMP-1 from NIH3T3 by ELISA



Effect of EPL, FEPL and Equol (50 μg/㎖) on (A) MMP-1, (B) MMP-3, (C) MMP-9 from NIH3T3 by ELISA



Conclusion

1. EtOH extract of *P. lobata* (EPL) and fermented of EPL (FEPL) up to 1000 μg/ml did not show any significant cell cytotoxicy on HaCaT, B16F10, HS68.
2. EPL showed remarkable antioxidant capacity but in the same amount of FEPL the antioxidant capacity was less than that of EPL.
3. EPL and FEPL could increase the production of Procollagen type I and elastin.
4. EPL could remarkably suppress the expression level of MMP-1, -3 and 9. In addition, FEPL showed more potent activity than that of EPL.
5. EPL and FEPL could significantly increase the protein expression level of TIMP-1.