A potential anti-inflammation activity and depigmentation effect of *Lespedeza bicolor* extract and its fractions

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**Abstract** Postinflammatory hyperpigmentation (PIH) is an acquire hypermelanosis after cutaneous inflammation and injury. The aim of the present study was to investigate a natural ingredient with the anti-inflammatory and depigmentation activities into possible applications of postinflammatory hyperpigmentation. Methanol extracts of *Lespedeza bicolor* and its various fractions inhibited LPS-induced NO production in RAW 264.7 macrophages in a concentration-dependent manner. In particular, the ethyl acetate fraction was shown to be inhibition of NO production (89%) and down-regulation of iNOS mRNA without causing cytotoxicity. In addition, ethyl acetate fraction significantly attenuated LPS-induced NF-κB activation (*P* < 0.05), indicating the anti-inflammatory activity due to NF-κB inhibition. Moreover, extracts, mainly ethyl acetate fraction, exhibited not only DPPH free radical scavenging activity (IC₅₀ 112.45 µg/mL) with 4 times lower activity than ascorbic acid, but also anti-tyrosinase activity (IC₅₀ 1 µg/mL) with a similar activity to arbutin showing a competitive inhibitor. Furthermore, vitexin and haginins A, B and C were identified through LC–MS analysis as potential compounds responsible for these effects. These results suggest that *L. bicolor* extract have anti-inflammatory, antioxidant activities and tyrosinase inhibitory effect and it might be used in the management of postinflammatory pigmentation through inhibition of pathogenic process involved in hyperpigmentation.

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1. Introduction

Postinflammatory hyperpigmentation (PIH) is an acquire hypermelanosis which induces skin color change occurring after cutaneous inflammation and injury (Davis and Callender, 2010). Although the exact mechanism is unknown, multiple studies have been shown to be a correlation between the inflammatory mediator released during the inflammatory process. Postinflammatory pigmentation is characterized by the accumulation of melanin granules in the basal layer of the epidermis, followed by the migration of melanocytes into the surrounding dermis. This process is mediated by various inflammatory mediators, including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and nitric oxide (NO). NO is produced by inducible nitric oxide synthase (iNOS), which plays a crucial role in the inflammatory response and is involved in the regulation of melanin synthesis. The inhibition of NO production by *Lespedeza bicolor* extract and its fractions suggests that it may have potential anti-inflammatory and depigmentation activities, which could be useful in the management of postinflammatory pigmentation.
process and melanocyte stimulation (Davis and Callender, 2010). Therefore, the treatment approach for PHI should be considerate in aspect of not only the inhibition of pigmentation but also improvement of underlying inflammatory dermatosis. 

*Lespedeza* species have been used as a traditional medicine for the treatment of acute and chronic inflammation of urinary tract in Chinese (Miyase et al., 1999). In the recent pharmaceutical field, *Lespedeza dichromatia* for new anti-ulcer agents and *Lespedeza canecata* for reduction of kidney damage and effect of vascular relaxation have been studied (Amosova et al., 2007). Nevertheless, the biological activities of *L. bicolor* have not been adequately investigated comparing with other *Lespedeza* species.

Hence, the current study was designed to elucidate an anti-inflammatory and depigmentation activity of *L. bicolor* extract whether it can be a potential material for a dermatologic and cosmetic applications for PHI.

2. Materials and methods

2.1. Compounds and reagents

All reagents were purchased from Sigma–Aldrich (USA) except luciferase reporter vectors such as pNFkB-Luc, pTAL-Luc and pTK-RL (Clontech Laboratories, USA).

2.2. Preparation of *L. bicolor* extract

Dried the stem of *L. bicolor* (200 g) was cut into small pieces and extracted with 70% methanol (1500 mL). After filtration of supernatant, methanol extract of *L. bicolor* (LME) was concentrated under reduced pressure using a rotary evaporator to dryness. LME (4.03 g) was re-suspended in water (400 mL) and fractionated using the Soxhlet apparatus with solvents of increase polarity: chloroform (CE), ethyl acetate (EE), n-butanol (BE) and water (WE). Each organic phase was later concentrated and dried dryness, yielding approximately 0.26, 0.28, 0.30 and 2.66 g of dried material, respectively. The remaining residues were dissolved in DMSO as a stock solution and then diluted serially with a medium in order to use as working solutions.

2.3. NO assay and cytotoxicity

RAW 264.7 cells (Korean Cell Line Bank, Seoul, Korea) maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂ humidified atmosphere. RAW 264.7 cells (4 × 10^5 cells) used in this experiment were allowed to adhere for 24 h until 80% confluency.

Cells were incubated with different fractions of LME at concentrations indicated for 18 h following LPS (0.5 μg/mL) treatment for 30 min. Cell medium and cell were used for NO assay and RT-PCR, respectively. NO production in cell medium was also measured colorimetrically as nitrite (NO₂⁻) at 540 nm using a multichannel spectrophotometer (VERSA max, Molecular Devices, Sunnyvale, CA, USA) and quantified from a standard curve generated with sodium nitrite (NaNO₂, 0–100 μM).

To check cytotoxicity by LME, RAW 264.7 cells treated with LME for 24 h incubation following by MTT assay (Kim et al., 2006). The absorbance of colored solution was quantified by measuring at 570 nm.

2.4. Reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cell with Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. One microgram of the RNA were subjected to RT reaction for cDNA generation using an AccuPower® RT PreMix (Bioneer, Daejeon, Korea), then, amplified using AccuPower® PCR Premix (Bioneer) in the presence of specific primers for iNOS (target) or β-actin (internal standard). The primer sequences are listed below: iNOS, 5’-CCCTTCCGAAGTTTCTGCGAG CAG-3′ (F) and 5’-GGCTGTCAAGGCTCTGGCTTTTG G-3′ (R); β-actin, 5’-ATGCTCTGCTGAGATGTATGT-3′ (F) and 5’-GGAGGAGGATGCGCGAGT-3′ (R). The PCR was performed in a thermal cycler system (MyCycler, Bio-Rad Laboratory, USA) under the following condition: 35 cycles each at 95 °C for 45 s (denaturation), 50 °C or 60 °C for 45 s (annealing), and 72 °C for 45 s (extension). Equal volumes of PCR products were fractionated on 1% agarose in 1× TAE stained with ethidium bromide, and visualized using EAGLE-EYE TM (Stratagene, La Jolla, CA, USA).

2.5. Transfection and luciferase reporter assay

RAW 264.7 cells were transfected with control and expression vectors using FuGENE® HD transfection reagent (Roche, Germany) according to the manufacturer’s instructions. Cells were co-transfected with pTK-RL (expression vector for *Renilla* luciferase) as a control. Transfected cells were treated with LME fractions in the presence of LPS (0.1 μg/mL) for 18 h. The luciferase activity of cell extract was then measured using a dual luciferase assay system (Promega, USA).

2.6. DPPH free radical scavenging activity

Scavenging activity of the extracts against DPPH free radicals was evaluated according to the previous method (Chang et al., 2008). Ascorbic acid was used as a positive control. The optical density was measured at 517 nm using microplate reader and the inhibition of samples on DPPH free radical was shown according to the following calculation formula: Inhibition (％) = (1 – ((Sd – Se)/C)) × 100％, where Sd is OD of sample with DPPH, Se is OD of sample with ethanol, and C is OD of vehicle with DPPH. The scavenging activity was showed IC₅₀ value which is the concentration of the sample required to inhibit 50% DPPH free radicals.

2.7. Tyrosinase inhibitory activity of LME

Tyrosinase inhibitory activity was determined according to a published method (Baek et al., 2008) and arbutin was used as a positive control. The inhibitory activity of the sample was calculated according to the following formula: inhibition
\[(\%) = \left[ \frac{(D - C) - (B - A)}{(D - C)} \right] \times 100\], where \(A\) and \(B\) are the OD\_492 nm values before and after the reaction in respect to the test samples, and \(C\) and \(D\) are the OD\_492 nm values before and after the reaction without the test samples. The Michaelis–Menten constant (\(K_m\)) and maximal velocity (\(V_{max}\)) of tyrosinase were determined by the Lineweaver–Burk plot with various concentrations of L-tyrosine as a substrate.

### 2.8. LC–MS analysis

LC–MS analysis was conducted on Agilent 1100 series liquid chromatograph system (Agilent, USA) with Varian 500MS (ESI-ion trap mode) mass spectrometer. A Varian, chromsep 150 \(\times\) 2.0 mm Pursuit XR-s 3 \(\mu\)m-C\(_{18}\) column was used with 0.2 mL/min of flow rate at 40 °C. The mobile phases used in this analysis consist of \(A\) (distilled water with 0.1% formic acid) and \(B\) (acetonitrile with 0.1% formic acid) and run according to a 60 min-programmed protocol. Compounds were detected by their absorption at 254 nm and ESI-MS.

### 2.9. Statistical analysis

All data were presented as mean ± SD (\(n = 3\)). Analysis for statistical difference was conducted by a one-way analysis of variance (ANOVA), Duncan’s, and Dunnett’s multiple comparisons test using Statistical Analysis System (SAS institute, USA). The level of statistical significance was set at \(P < 0.05\).

### 3. Results

#### 3.1. Effect of LME on NO production and cytotoxicity

In the present study, the anti-inflammatory activity of LME was estimated in vitro model with LPS-stimulated RAW 264.7 cells. LME (0–200 \(\mu\)g/mL) inhibited LPS-induced NO production in a concentration-dependent manner, showing a significant inhibition (78%) at maximum concentration (data not shown). To examine the potential cell cytotoxicity by LME, MTT assay was conducted. LME treatment significantly increased the cell viability (83%) during LPS stimulation compared to LPS alone (71%) (data not shown). The result indicates that the inhibitory effect of LME on NO production was caused by cytotoxic effect by LME.

The effect of various solvent fractions of LME, including CE, EE, BE and WE, on LPS-induced NO production and cell viability were further evaluated. As shown in Fig. 1A, both CE and EE (50 \(\mu\)g/mL) inhibited NO production by 85% and 89%, respectively in LPS-stimulated cells, but in CE fraction, significant effect of cytotoxicity was showed (Fig. 1B). Hence, we selected EE among LME fraction for the subsequent experiment.

#### 3.2. Effect of EE fraction on NO production and iNOS gene expression level

Exposure of RAW 264.7 cells to the EE fraction (6.25–50 \(\mu\)g/mL) of LME inhibited LPS-induced NO production in a concentration-dependent manner (Fig. 2A). This finding was further confirmed to correlate with inhibition of mRNA level by EE fraction of LME using RT-PCR analysis. Similarly, EE fraction suppressed a concentration-dependent increase in iNOS gene expression by LPS (Fig. 2B) in RAW 264.7 cells, showing a positive correlation between inhibition of NO production and suppression of iNOS mRNA by EE fraction of LME.

#### 3.3. Effect of LME on NF-\(\kappa\)B activity

To elucidate the mechanism underlying the inhibitory effect of the EE fraction on inflammatory response, NF-\(\kappa\)B activity was evaluated by luciferase reporter assay. The NF-\(\kappa\)B promoter activity under resting condition was approximate 7% compared to cells stimulated with LPS (set at 100%), and the EE fraction significantly attenuated the effect of LPS on gene expression through NF-\(\kappa\)B inhibition (Fig. 2C).

#### 3.4. DPPH free radical scavenger activity of LME

To determine the antioxidant activity of LME, DPPH assay was conducted. A dose response was observed for all fractions of LME on the scavenging effects of DPPH free radicals (Fig. 3). The IC\(_{50}\) value of EE, BE, ME, CE and WE were
112.45, 141.01, 209.65, 340.97 and 687.31 µg/mL, which were about 4–20 times higher than those obtained with ascorbic acid (31.77 µg/mL).

3.5. Anti-tyrosinase activity of LME

In the analysis of tyrosinase inhibitory activity by LME, all fractions inhibited tyrosinase activity in a concentration-dependent manner with the IC$_{50}$ value of 136.1 µg/mL (CE), 1.0 µg/mL (EE), 1107.8 µg/mL (BE), 16384.19 µg/mL (WE) and 1.0 µM (arbutin) (Fig. 3). EE showed the greatest inhibitory effect on tyrosinase activity among fractions, in particular, the IC$_{50}$ of EE was similar with that of arbutin used as a positive control. Subsequently, the kinetic study of tyrosinase was performed after exposure to the EE and analyzed by Lineweaver–Burk plots. As shown in Fig. 4, EE increased the $K_m$ value of mushroom tyrosinase activity with no change in the $V_{max}$ value ($\Delta A_{492}$/min). This result indicated that the EE is a competitive inhibitor of mushroom tyrosinase.

3.6. LC–MS analysis of compounds of LME

To identify the compound(s) responsible for the physiological effects of LME, the EE fraction was analyzed using LC–MS, which resulted in the identification of vitexin and haginins A, B and C (Fig. 5). In particular, Vitexin, a natural flavonoid, is one of the major components of the EE fraction from LME.

4. Discussion

The aim of the present study was to confirm the potential activity of anti-inflammatory and depigmentation of LME used in traditional medicine for the further dermatologic and cosmetic applications for PHI. Since melanocyte activity has been shown to be stimulated by inflammatory mediators that are released during the inflammatory process such as melanocyte-stimulating properties of leukotrienes (LT), prostaglandins E2 and D2, thromboxane-2, interleukin-1 (IL-1), IL-6,
EE fraction of LME could suppress other pro-inflammatory cytokine such as IL-1β, IL-6 and TNF-α through NF-κB inhibition, which is a key signal pathway in inflammatory response. Therefore, EE fraction of LME might be used as a therapy agent for PIH and diverse inflammation disease by reducing production of pro-inflammatory cytokines through further study.

Antioxidants are used in the treatment for PIH as a supplementary agent because of their ability to increase efficacy of major treatment such as hydroquinone monotherapy. Ascorbic acid, a represented antioxidant, has been reported not only skin lightening but also anti-inflammatory have been reported (Davis and Callender, 2010). In the present study, all fractions of LME exhibited a similar level of DPPH free radical scavenger capacity. EE fraction was shown to have the greatest effect (IC₅₀, 112.45 µg/mL) among them, which is lesser than that of ascorbic acid (3.5-fold) or a respective compound isolated from Lespedeza cyrtobotrya (2- or 4-fold) (Baek et al., 2008). The antioxidant effect of LME could affect to directly reduce reactive oxygen species and indirectly has a synergic effect with major treatment in therapy for PIH.

Melanin pigments result from distinct reactions of melanin synthesis by tyrosinase catalysis, the hydroxylation of a monophenol and the conversion of an o-diphenol to the corresponding o-quinone (Chang et al., 2008). Thus, for lighten areas of hypermelanosis, hydroquinone, azelaic acid, kojic acid, arbutin and certain licorice extracts were used effectively as a tyrosinase inhibitors (Davis and Callender, 2010). Hence, the anti-tyrosinase activity of LME was further estimated. EE fraction was similar to the anti-tyrosinase activity (IC₅₀ of 1.0 µM) of arbutin, a positive control, showing a competitive inhibitor of mushroom tyrosinase. To be compared with licorice extracts which is used in topical depigmentation agent, EE fraction was also showed similar effect (Nerya et al., 2003). Moreover gallic acid, which is well known as a competitive inhibitor of tyrosinase (De Melo et al., 2005; Picerno et al., 2006; Coelho et al., 2008) and a represented antioxidant, has been reported not only skin lightening but also anti-inflammatory have been reported (Davis and Callender, 2010). Therefore, it is likely that vitexin in LME may be responsible for the biological activities. Haginins, mainly B and C, were also isolated from the EE extract. Although not much is known regarding its biological activity, a recent report suggested that haginin A can decrease hyperpigmentation caused by UV irradiation or skin disorders through down-regulation of tyrosinase and tyrosinase-related protein-1 (TRP-1) production (Kim et al., 2008). The compounds isolated in our study may be responsible for the anti-tyrosinase activity of L. bicolor. However, a further investigation is required to quantify and determine the exact role and activity of each compound.

5. Conclusions

In conclusion, our results demonstrate that different fractions from LME, mainly the EE fraction is capable of inhibiting NO production by inhibition of NF-κB and oxidative damages.
Also, vitexin and haginins from EE fraction were identified as potential compounds responsible for these effects. Our findings suggest that LME, especially EE fraction might have a potential effect on the treatment with anti-inflammatory and antioxidant activities as well as tyrosinase inhibitor for the prevention or treatment of PIH.

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References

Amosova, E.N., Zueva, E.P., Razina, T.G., Turetskova, V.F., Azarova, O.V., Krylova, S.G., Goldberg, E.D., 1998. The search for new anti-aleur agents from plants in Siberia and the Far East. Eksp. Klin. Farmakol. 61, 31–35.

Baek, S.H., Kim, J.H., Kim, D.H., Lee, C.Y., Kim, Y.J., Chung, D.K., Lee, D.H., 2008. Inhibitory effect of dalbergioidin isolated from the trunk of Lespedeza cyrtoforiya on melanin biosynthesis. J. Microbiol. Biotechnol. 18, 874–879.

Chang, Y.H., Lee, S.T., Lin, W.W., 2001. Effects of cannabinoids on lipopolysaccharide-stimulated macrophages RAW 264.7. Biochem. Pharmacol. 61, 195–202.

Eksp. Klin. Farmakol. 61, 31–35.

Kim, J.H., Baek, S.H., Kim, D.H., Choi, T.Y., Yoon, T.J., Hwang, J.S., Kim, M.R., Kwon, H.J., Lee, C.H., 2008. Downregulation of melanin synthesis by haginin A and its application to in vivo lightening model. J. Invest. Dermatol. 128, 1227–1235.

Kim, J.H., Lee, B.C., Kim, J.H., Sim, G.S., Lee, D.H., Lee, K.E., Yun, Y.P., Pyo, H.B., 2005. The isolation and anti-inflammatory effects of vitexin from Acer palmatum. Arch. Pharm. Res. 28, 195–202.

Kim, J.K., Oh, S.M., Kwon, H.S., Oh, Y.S., Lim, S.S., Shin, H.K., 2006. Anti-inflammatory effect of roasted licorice extracts on lipopolysaccharide-induced inflammatory responses in murine macrophages. Biochem. Biophys. Res. Commun. 345, 1215–1223.

Lee, J.K., Kang, D.G., Lee, H.S., 2012. Vascular relaxation induced by aqueous extract of Lespedeza cuneata via the NO-cGMP pathway. J. Nat. Med. 66, 17–24.

Maximov, O.B., Kulesh, N.I., Stepanenko, L.S., Dmitrenok, P.S., 2004. New prenylated isoflavonanes and other constituents of Lespedeza bicolor. Fitoterapia 75, 96–98.

Miyase, T., Sano, M., Nakai, H., Muraoka, M., Nakazawa, M., Suzuki, M., Yoshino, K., Nishihara, Y., Tanai, J., 1999. Antioxidants from Lespedeza homolobia (I). Phytochemistry 52, 303–310.

Na, H.J., Lee, G., Oh, H.Y., Jeon, K.S., Kwon, H.J., Ha, K.S., Lee, H., Kwon, Y.G., Kim, Y.M., 2006. 4-O-methylgallic acid suppresses inflammation-associated gene expression by inhibition of redox-based NF-kappaB activation. Int. Immunopharmacol. 6, 1597–1608.

Nerya, O., Vaya, J., Musa, R., Israel, S., Tamir, S., 2003. Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. J. Agric. Food Chem. 51, 1201–1207.

Ortonne, J.P., Bissett, D.L., 2008. Latest insights into skin hyperpigmentation. J. Invest. Dermatol. Symp. Proc. 13, 10–14.

Park, Y.M., Won, J.H., Kim, Y.H., Choi, J.W., Park, H.J., Lee, K.T., 2005. In vivo and in vitro anti-inflammatory and anti-nociceptive effects of the methanol extract of Inonotus obliquus. J. Ethnopharmacol. 101, 120–128.

Pereira, C.A.M., Yariwake, J.H., Lancas, F.M., Santos, J.N., Tiss, M., Angenot, L., 2004. A HPTLC densitometric determination of flavonoids from Passiflora alata, P. edulis, P. incarnata and P. caerulea and comparison with HPLC method. Phytochem. Anal. 15, 241–248.

Picerno, P., Mencherini, T., Della Loggia, R., Meloni, M., Sanogo, R., Aquino, R.P., 2006. An extract of Lannea microcarpa: composition, activity and evaluation of cutaneous irritation in cell cultures and reconstituted human epidermis. J. Pharm. Pharmacol. 58, 981–988.

Tan, L., Zhang, X.F., Yan, B.Z., Shi, H.M., Du, L.B., Zhang, Y.Z., Wang, L.F., Tang, Y.L., Liu, Y., 2007. A novel flavonoid from Lespedeza virgata (Thunb.) DC: structural elucidation and antioxidative activity. Bioorg. Med. Chem. Lett. 17, 6311–6315.

Tomita, Y., Hase, K., Tagami, H., 1992. Melanocyte-stimulating hormone factor-2005. Down-regulatory effect of quercitrin gallate on nuclear factor of activated T cells (NF-ATC) in vitro. J. Ethnopharmacol. 101, 120–128.

Wang, C.Y., Deng, H.Z., Li, H., 2005. Experimental study on treatment of minimal change nephropathy with Lespedeza bicolor. Zhongguo Zhong Yao Za Zhi 30, 614–617.