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ORIGINAL ARTICLE

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 (IC_{50}, 112.45 μg/mL) with 4 times lower activity than ascorbic acid, but also anti-tyrosinase activity (IC_{50}, 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.

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

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process and melanocyte stimulation (Davis and Callender, 2010). Therefore, the treatment approach for PIH 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 dichromatic* for new anti-ulcer agents and *Lespedeza cuneata* for reduction of kidney damage and effect of vascular relaxation have been studied (Amosova et al., 1998; Lee et al., 2012; Wang et al., 2005). Also, various compounds including ethyl caffeate, caffeic acid, betulinic acid, betulin, β-sitosterol, protocatechuic acid, and more recently two prenylated isoflavanones have been identified from the stem bark of *Lespedeza bicolor* (Maximov et al., 2004; Tan 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 pNFκB-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% CO_2 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 (NO_2^-) at 540 nm using a multichannel spectrophotometer (VERSA max, Molecular Devices, Sunnyvale, CA, USA) and quantified from a standard curve generated with sodium nitrite ($NaNO_2$, 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′-CCCTTCCGAAGTTTCTGGCAG CAG-3′ (F) and 5′-GGCTGTCAGAGCCTCGTGGCTTTG G-3′ (R); β-actin, 5′-ATGCTCCTGCTTGAGTATGT-3′ (F) and 5′-GGAGGAAGAGGATGCGGCAGT-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 (%) = $(1 - ((Sd - Se)/C)) \times 100\%$, 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 IC_{50} 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

(%) = $[(D - C) - (B - A)]/(D - C) \times 100$, where A and B are the OD_{492nm} values before and after the reaction in respect to the test samples, and C and D are the OD_{492nm} 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 × 2.0 mm Pursuit XRs-3 μm-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-programed 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 μg/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 μg/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 μg/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,

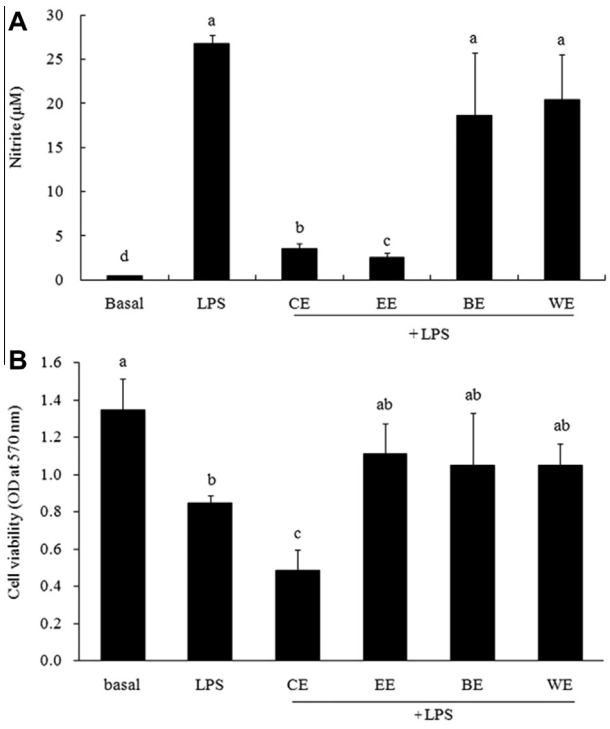

Figure 1 Effects of different fractions from methanol extract of *L. bicolor* (LME) on LPS-induced NO production (A) and cell viability (B). Cells were pretreated with chloroform fraction (CE), ethyl acetate fraction (EE), butanol fraction (BE) and water fraction (WE) from methanol extract of *L. bicolor* at 50 μg/mL concentration for 30 min and activated with LPS (0.5 μg/mL). After incubation for 24 h, (A) the supernatant was collected and the nitrite amounts determined by using the Griess reagent. (B) Cells were further exposed to MTT in fresh medium for 4 h, and cell viability determined by MTT assay. Data represent the mean ± SD of three independent experiments. Bars with different letters show significant differences ($P < 0.05$).

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-κB activity

To elucidate the mechanism underlying the inhibitory effect of the EE fraction on inflammatory response, NF-κB activity was evaluated by luciferase reporter assay. The NF-κB 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-κB 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

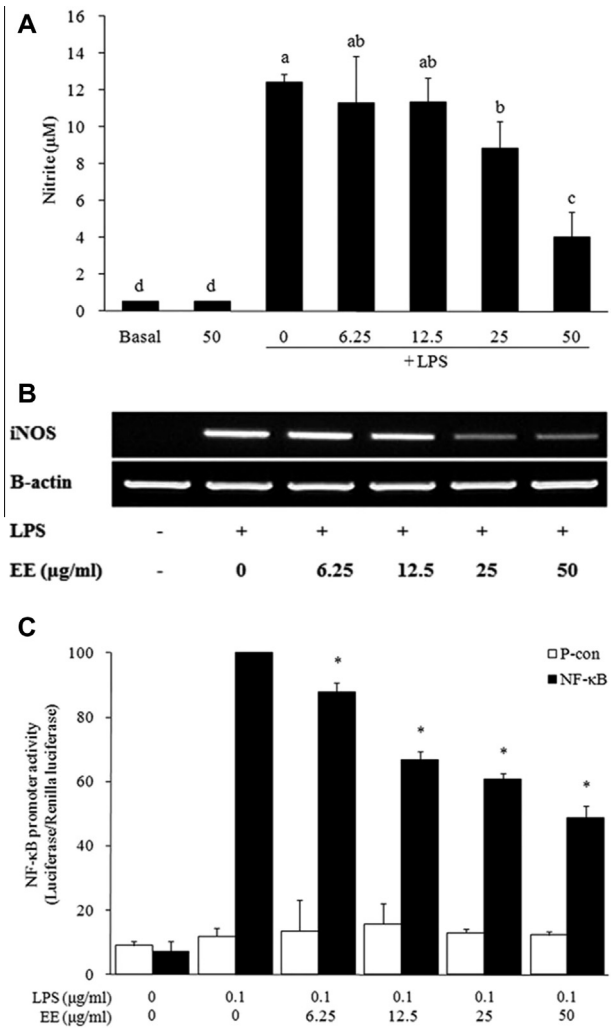

Figure 2 Effect of ethyl acetate (EE) fraction from methanol extract of *L. bicolor* on LPS-induced NO production (A), iNOS mRNA expression (B), and activity of NF-κB promoter (C). Cells were pretreated with ethyl acetate fraction from methanol extract of *L. bicolor* at different concentrations (0–50 μg/mL) for 30 min and then activated with LPS (0.5 μg/mL). After incubation for 18 h, (A) the medium supernatant was collected and the nitrite amounts determined using Griess reagent. (B) iNOS mRNA from cell lysate was assessed by semi-quantitative RT-PCR. Representative images of PCR products of two independent experiments are shown. The β-actin was used as an internal control for equal loading. (C) RAW264.7 macrophages were co-transfected with NF-κB promoter luciferase and *Renilla* luciferase reporters and stimulated with LPS (0.1 μg/mL) in the presence or absence of *L. bicolor* ethyl acetate fraction (EE) for 24 h. pTAL-Luc vector was used as a negative control to determine background signals. Cell lysates were prepared and luciferase activity measured using the Dual-Luciferase Reporter Assay System. Data represent the mean ± SD of three independent experiments. $^{*}P < 0.05$ compared to LPS treated control.

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 (ΔA_{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, interlukin-1 (IL-1), IL-6,

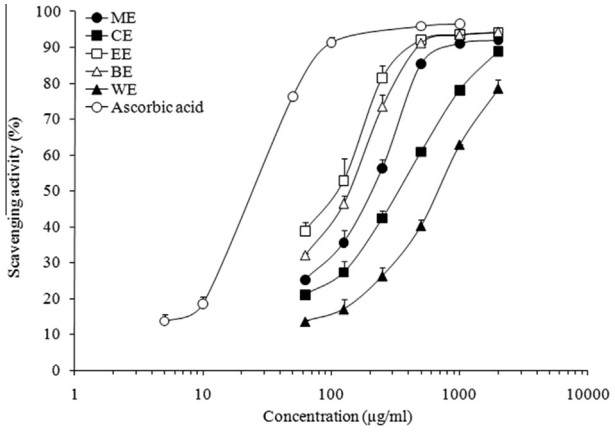

Figure 3 Scavenging activities of a various fractions from methanol extract of *L. bicolor* for DPPH free radical. Antioxidant activity of a various fractions from methanol extract of *L. bicolor* was evaluated by using DPPH free radical solution. After incubating at 37 °C for 30 min, the optical density was measured at 517 nm. Ascorbic acid was used as a positive control for antioxidant activity. ME, methanol extract of *L. bicolor*; CE, chloroform fraction; EE, ethyl acetate fraction; BE, butanol fraction; WE, water fraction.

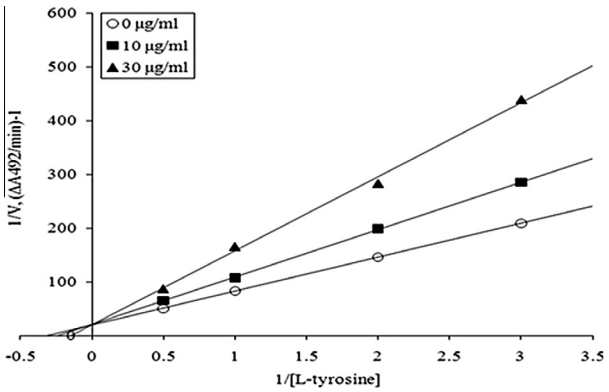

Figure 4 Lineweaver–Burk plot of tyrosinase in the presence EE fraction of *L. bicolor*. Data were obtained as mean values of $1/V$, inverse of the increase in absorbance at a wavelength of 492 nm per min (ΔA_{492}/min), with different concentrations (0, 10, 30 μg/mL) of tyrosine as a substrate.

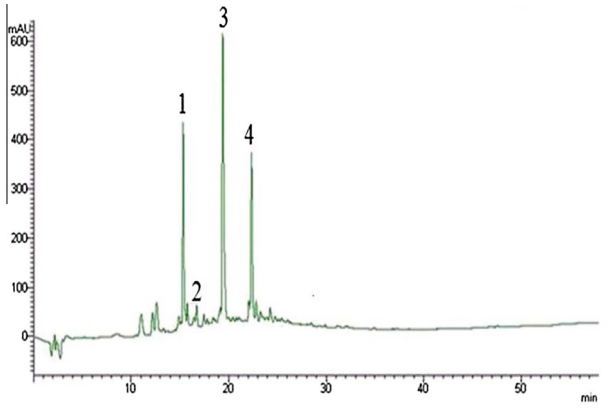

Figure 5 LC chromatogram of EE fraction from methanol extract of *L. bicolor* at 254 nm. Peaks; 1, Vitexin; 2, Haginin A; 3, Haginin C; 4, Haginin B.

tumor necrosis factor (TNF) epidermal growth factor, and reactive oxygen species such as NO (Tomita et al., 1992; Ortonne and Bissett, 2008). Therefore, to control inflammation is useful for therapeutic purpose of PHI. In order to elucidate anti-inflammatory effect of LME and its fractions, *in vitro* model with LPS-stimulated RAW 264.7 cells was used in present study. It is well established that LPS, cell wall of gram negative bacteria, activates a series of intracellular signal pathways and consequent production of cytokines (IL-1β, IL-6 and TNF-α) and mediators (NO and PGE_2) in monocytes/macrophages (Chang et al., 2001). We found that EE fraction of LME significantly inhibited LPS-induced NO production and iNOS mRNA expression through attenuation of NF-κB activity in RAW 264.7 cells. Some plant extracts and mushroom were also exhibited same effect on LPS-induced NF-κB (Kim et al., 2006; Park et al., 2005). LME, especially EE fraction suppressed LPS-induced NO production and iNOS mRNA expression through attenuation of NF-κB activity without cytotoxicity in RAW 264.7 cells. Although LPS-induced NO, a representative inflammatory mediator, was focused in this study as an indicator for anti-inflammatory effect, the experimental evidence from our study suggests that 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 (IC_{50}, 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 (IC_{50} 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 (IC_{50} of 4.5 μM), EE fraction was showed 4500-fold higher effect (Na et al., 2006). The results indicated that EE fraction may be more useful to prevent enzymatic oxidation than not-enzymatic oxidation.

Vitxein was isolated from LME. It has been isolated from other *Lespedeza* species as well as other plants showing antioxidant, anti-inflammatory, anti-ulcer and anti-cancer activities (De Melo et al., 2005; Picerno et al., 2006; Coelho et al., 2008; Pereira et al., 2004; Kim et al., 2005; Choi et al., 2006). 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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