Original article

Lespedeza bicolor ameliorates endothelial dysfunction induced by methylglyoxal glucotoxicity

Moon Ho Do, Jae Hyuk Lee, Hussain Mustatab Wahedi, Chaeho Pak, Choong hwan Lee, Eui-Ju Yeo, Yunsook Lim, Sang Keun Ha, Inwook Choi, Sun Yeou Kim

A B S T R A C T

Background: Lespedeza species have been used as a traditional medicine to treat nephritis, azotemia, inflammation, energy depletion, diabetes, and diuresis. The purpose of this study is to screen the most potent Lespedeza species against methylglyoxal (MGO)-induced glucotoxicity, and to elucidate the mechanisms of action. Also, we will attempt to identify small chemical metabolites that might be responsible for such anti-glucotoxicity effects.

Methods: Firstly, the protective effect of 26 different Lespedeza species against MGO-induced toxicity in human umbilical vein endothelial cells was investigated. The chemical metabolites of the most potent species (Lespedeza bicolor) were identified by high pressure liquid chromatography quadrupole time-of-flight tandem mass spectrometry (HPLC-Q-TOF-MS/MS), then quantified by HPLC. The effects of LB1 on MGO-induced apoptosis were measured by annexin V-FITC staining and western blot. Inhibitory effects of LB1 on MGO-induced ROS generation, and effect of LB1 on advanced glycation end products (AGES) inhibitor or a glycated cross-link breaker are also measured.

Results: Among different Lespedeza species, LB1 extract was shown to reduce intracellular reactive oxidative species, exhibit anti-apoptotic effects, strongly inhibit all the mitogen-activated protein kinase signals, inhibit reactive oxidative species, and ultimately cell death in many cell types (Yamagishi, 2011). A highly reactive AGEs precursor methylglyoxal (MGO) can be formed either by enzyme-catalyzed steps or by non-enzymatic reactions (Vistoli et al., 2013). MGO-induced carbonyl stress may induce cellular damage by glycation, cross-linking of proteins, and ultimately cell death.

Conclusion: LB1 extract has shown to be effective in preventing or treating MGO-induced endothelial dysfunction.

Introduction

Long-term hyperglycemic conditions associated with diabetes lead to the formation of advanced glycation end products (AGEs). Interaction between AGEs and their receptor might induce oxidative stress, inflammatory reactions, and thrombosis which can cause vascular aging, retinopathy, atherosclerosis, nephropathy, generation of reactive oxygen species (ROS), and ultimately cell death in many cell types (Yamagishi, 2011). A highly reactive AGEs precursor methylglyoxal (MGO) can be formed either by enzyme-catalyzed steps or by non-enzymatic reactions (Vistoli et al., 2013). MGO-induced carbonyl stress may induce cellular damage by glycation, cross-linking of proteins, and ultimately cell death.

Keywords:
Advanced glycation end products (AGEs)
Methylglyoxal (MGO)
Human umbilical vein endothelial cells
Lespedeza bicolor (LB)
Apoptosis
 Reactive oxygen species (ROS)
proteins, cytotoxicity, ROS generation, and mitogen-activated protein kinase (MAPK) signaling cascade (Figarola et al., 2014). MGO-induced AGEs can produce endothelial dysfunction in diabetic individuals and can increase the chances of vascular diseases (Sena et al., 2012). AGEs inhibitor or a glycated cross-link breaker may have a broad-range impact on the treatment of diabetes related complications.

Lespedeza species (Leguminosae) which are widely distributed in Eastern North America and Eastern Asia (Sun et al., 2016) have been used as a traditional medicine to treat nephritis, azotemia, inflammation, hyperpigmentation, energy depletion, diabetes, and diuresis (Maximov et al., 2004). Recent studies on these species have also shown effectiveness in treating cough and fever, as well as having estrogenic effects and inhibitory effects on xanthine oxidase and tyrosinase (Lee et al., 2006). It is interesting to note that insufficient amount or action of estrogen may cause obesity, insulin resistance and vascular relaxation (Lee et al., 2012). Therefore Lespedeza species have the potential to be used as estrogen alternative for improving obesity and diabetic complications. In the present study, firstly, 26 different Lespedeza species are tested for anti-glucotoxicity effects on human umbilical vein endothelial cells (HUVECs). And, we are to investigate the molecular mechanisms of anti-apoptotic effect of one of Lespedeza species, Lespedeza bicolor 1 (LB1), which has shown to have the highest effectiveness on glucotoxicity. Our study focused on MS-based identification of small metabolites from the stalk of LB1 using high pressure liquid chromatography quadrupole time-of-flight tandem mass spectrometry (HPLC-Q-TOF-MS/MS) followed by their anti-glucotoxicity effects.

Materials and methods

Materials

MGO, aminoaguandine (AG), 2’,7’-dichlorofluorescein diacetate (DCF-DA), genistein, quercetin and naringenin were purchased from Sigma (St. Louis, MO, USA). Daidzein was obtained from LC Resources (Schaumburg, IL, USA). Antibodies against p38 (cat no. 9212S), phospho-p38 (cat no. 9211S), extracellular signal-regulated kinase (ERK, cat no. 9102S), phospho-ERK (cat no. 9101S), c-Jun N-terminal kinases (JNK, cat no. 9252S), and phospho-JNK (cat no. 9251S) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies against α-tubulin (cat no. sc-5286), β-cell lymphoma 2 (Bcl-2, cat no. sc-492) and Bcl-2-associated X protein (Bax, cat no. sc-493) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of standard solutions and sample extracts

Standard stock solutions of genistein, daidzein, quercetin and naringenin were prepared by dissolving 1 mg of each compound in 1 mL methanol and stored at −20 °C. The stalks of Lespedeza species were obtained and authenticated by Dr. Choong Hwan Lee (Konkuk University), and voucher specimens (KSY-HP-008 - 033) were deposited at the College of Pharmacy in Gachon University. Lespedeza species were extracted with 70% ethanol at room temperature overnight. Afterwards, the extract was filtered and evaporated. The extract was dissolved in methanol at a concentration of 10 mg/mL.

HPLC-Q-TOF-MS/MS analysis conditions

HPLC-Q-TOF-MS/MS analysis was performed on the Agilent series 1290 Infinity HPLC instrument (Agilent, Waldbronn, Germany) coupled with an Agilent 6530 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray (ESI) interface. Chromatographic separation was carried out on Hydrosphere C18 (150 × 3.0 mm; particle size, 3 um; YMC Co. Ltd., Japan) with 0.1% formic acid-water (v/v, solvent A) and acetonitrile (solvent B) as the mobile phase, and column temperature was maintained at 30 °C with a flow rate of 0.6 mL/min. The gradient profile was 0–25 min, 80–0% A, 25–30 min, 100% A. For MS detection, the other optimum values of the source parameters were as follows: drying gas flow rate, 5.0 L/min; drying gas temperature, 325 °C; nebulizer, 20 psi; capillary, 4000 V; and fragment or voltage, 175 V. The sample collision energy was set at 15–45 V. The structural characterizations of the components were based on fragmentation pattern, previous reports and data from mass bank (http://www.massbank.jp).

HPLC analysis

To confirm the components of the LB1, HPLC analysis was performed on the Waters system (Waters Corp., Milford, MA, USA), consisting of a separation module (e2695) with a photodiode array detector (2998). Qualitative analysis was carried out at 283 nm. LB1 and its components were analyzed using an YMC-Triart C18 (250 × 4.6 mm; particle size, 5 μm; YMC Co. Ltd., Japan) with 0.1% phosphoric acid-water (v/v, solvent A) and acetonitrile (solvent B) as the mobile phase, and column temperature was maintained at 30 °C with a flow rate of 1 mL/min. The gradient was 0.0 min, 93% A; 50.0 min, 50% A.

Cell culture

HUVECs were obtained from the American Type Culture Collection (Lot # 60319874, ATCC, VA, USA). HUVECs were cultured under standard cell culture conditions (37 °C in a humidified incubator containing 5% CO2) in EGM-2 supplemented with 4% FBS. The passage number of all the cells used was between 5 and 8.

Measurement of cell viability

The 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT) assay was performed to measure cell viability. MGO and AG treatment concentration was chosen according to the suggestions of Figarola et al. with slight modifications (Figarola et al., 2014). HUVECs were briefly seeded at 1.0 × 104 cells/well in 96-well plates and incubated for 24 h. The cells were then pretreated with or without LB1 and AG for 1 h, followed by treatment with MGO for 24 h. After incubation, MTT solution was added at a final concentration of 0.1 mg/mL. This was followed by a 2 h incubation. Then, medium was removed and dimethyl sulfoxide (100 μL/well) was added. The absorbance at 570 nm was measured using a microplate reader (Molecular Devices, CA, USA). Cell morphological change of HUVECs was observed using IncuCyte ZOOM™ (Essen BioScience, Ann Arbor, MI, USA).

Western blotting analysis

Changes in the levels of proteins related to MAPK and apoptosis in the HUVECs were evaluated by western blot. Total proteins were extracted using PRO-PREP (iNtRON Biotechnology, Seongnam, Korea) containing phosphatase inhibitor. Equal amounts of protein were loaded and then transferred onto a nitrocellulose membrane. Membranes were blocked with 5% skim milk for 1 h at room temperature and incubated with the primary antibodies overnight at 4 °C. In all conditions, the primary antibodies were diluted 1:1000. Chemiluminescence was detected with ECL reagents using a ChemiDoc XRS+ imaging system (Bio-Rad, CA, USA).

Cell apoptosis assay

An Annexin V-FITC kit (Santa Cruz Biotechnology, CA, USA) was used to determine the effect of LB1 on MGO-induced apoptosis in HUVECs. 5.0 × 105 cells were seeded onto a 6-well plate and incubated overnight at 37 °C. The cells were then treated with MGO and LB1 for 24 h. After incubation, cells were stained for 15 min at room
temperature with Annexin V-FITC and propidium iodide (PI) in binding buffer and then analyzed by flow cytometry (FACScanibur flow cytometer; Becton Dickinson, San Jose, CA).

Detection of intracellular ROS

The intracellular ROS scavenging activity of LB1 was measured using DCF-DA. Briefly, 2.0 × 10^5 cells were seeded in a 12-well plate. After 24 h, cells were pre-incubated with LB1 for 1 h, followed by incubation with MGO for 2 h. Cells were washed with PBS, then 10 μM DCF-DA was added. The cells were then incubated for 20 min at 37 °C and washed with PBS. Cells were photographed using a Jujli live-cell imaging system (NanoEnTek, Seoul, Korea).

Inhibitory effects of LB1 on AGEs formation

The AGEs formation assay was used for investigation of inhibition in the middle stage of the glycation process according to Kiho et al. (Kiho et al., 2005). The formation of AGEs was determined using fluorescence at an excitation/emission wavelength of 355/460 nm with a VICTOR™ X3 multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) and values are given in mean ± S.D. The statistical results were further analyzed by one-way ANOVA and Bonferroni’s test. A p-value < 0.05 was considered statistically significant.

Results

Effects of Lespedeza genus on MGO-induced cytotoxicity in HUVECs

To choose the Lespedeza species with the most protective effects against MGO-induced damage, we performed cell screening for 26 specimens using MTT, and we chose LB1 with the lowest IC50 value (Table 1 and Table S1). As shown in Fig. 1A, treatment with LB1 ameliorated MGO-induced morphological changes compare to the cells treated with MGO only. MTT assay was performed to quantify the protective effects of LB1 on MGO-induced cytotoxicity of HUVECs. MGO treatment significantly reduced the cell viability of HUVECs but with the pretreatment with LB1 markedly reversed cytotoxic effects in a dose-dependent manner. (Fig. 1B).

Effects of LB1 on MGO-induced apoptosis in HUVECs

To examine whether MGO-induced cell death is related to apoptosis, we used fluorescence activated cell sorting analysis based on annexin V-FITC and PI double staining. As shown in Fig. 2A, MGO-treated HUVECs showed an increase in the number of both early and late apoptotic cells. However, with the pretreated LB1, these apoptosis was reversed in a dose-dependent manner (Fig. 2B).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell viability (%)</th>
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<th>Cell viability (%)</th>
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<td>MGO only</td>
<td>100.00 ± 3.78</td>
<td>L. cyrtobotrya 7</td>
<td>118.00 ± 1.68**</td>
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<td>L. bicolor 1</td>
<td>139.79 ± 4.78***</td>
<td>L. cyrtobotrya 8</td>
<td>112.39 ± 5.65</td>
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<tr>
<td>L. bicolor 2</td>
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<td>135.35 ± 0.82***</td>
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<td>132.04 ± 3.75***</td>
<td>L. bicolor 2 (China)</td>
<td>121.25 ± 3.64***</td>
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<tr>
<td>L. bicolor 4</td>
<td>128.49 ± 2.97***</td>
<td>L. bicolor 3 (China)</td>
<td>131.03 ± 4.77***</td>
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<td>L. bicolor 5</td>
<td>129.43 ± 2.73***</td>
<td>L. cuneate 1</td>
<td>107.59 ± 2.69</td>
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<tr>
<td>L. bicolor 6</td>
<td>135.06 ± 7.83***</td>
<td>L. cuneate 2</td>
<td>111.15 ± 6.19</td>
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<tr>
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<td>L. maximowiczii 1</td>
<td>97.33 ± 3.23</td>
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<tr>
<td>L. cyrtobotrya 1</td>
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<td>L. maximowiczii 2</td>
<td>115.94 ± 7.27</td>
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<td>L. cyrtobotrya 2</td>
<td>139.73 ± 2.05***</td>
<td>L. maximowiczii 3</td>
<td>115.31 ± 3.024</td>
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<tr>
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<tr>
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<td>L. maximowiczii 6</td>
<td>118.02 ± 8.50***</td>
</tr>
<tr>
<td>L. cyrtobotrya 6</td>
<td>112.34 ± 4.40</td>
<td>AG*</td>
<td>147.39 ± 2.09***</td>
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</table>

Values are presented as mean ± standard deviation of n = 3 experiments. *P, 0.05, **P, 0.01, ***P, 0.001 vs. respective MGO only group. * Positive control is in bold.

Effects of LB1 on MGO-induced expression of apoptosis-related proteins and activation of MAPK cascade

Using western blot analysis, we investigated whether MGO and pretreated LB1 had any effects on the expression of Bax, p53, Bcl-2 proteins, and the activation of MAPKs (p38, ERK, and JNK) in HUVECs. As shown in Fig. 3, when pretreated LB1 down regulated the expressions of Bax and p53, and increased the expression of Bcl-2 (Fig. 3A-D), MGO enhanced Bax and p53 protein expression (Fig. 3A, C, and D) and decreased Bcl-2 (Fig. 3A and B). In addition, pretreated LB1 blocked the activation of all three MAPKs, when MAPKs were increased in MGO-treated HUVECs (Fig. 3E-H).

Effects of LB1 on MGO-induced intracellular ROS levels

To investigate the molecular mechanisms of anti-apoptotic effect of LB1, we measured the intracellular ROS levels using fluorescence microscopy. As shown in Fig. 4, MGO-induced fluorescence enhancement was blocked by pretreated LB1 as an indication of intracellular ROS reduction in a dose-dependent manner.

Effects of LB1 on the formation and breakage of AGEs

We performed an AGEs formation assay by measuring fluorescence using AG as a positive control. As shown in Fig. 5A, following BSA-MGO incubation, the formation of AGEs was significantly increased. However, addition of LB1 (5–10 mg/mL) significantly inhibited the formation of AGEs in a dose-dependent manner (Fig. 5A).

To investigate whether LB1 had the ability to break the preformed AGEs, TNBS assay was carried out to measure the amounts of remaining glycation. MGO-BSA reaction significantly reduced free amines (Fig. 5B). Our results showed that LB1 increased the number of free amines, restoring the amine content of MGO-BSA to 16 to 23% after 24 h resulting in the breakage of AGEs. LB1 not only inhibited AGEs formation, but also broke down MGO-generated AGEs in a dose-dependent manner (Fig. 5B).

**MS-based small metabolite identification from the stalk of LB1 using HPLC-Q-TOF-MS/MS**

To identify small chemical metabolites in the stalk of LB1, an HPLC-Q-TOF-MS/MS analysis was used. Fig. 6A shows the HPLC-Q-TOF-MS/MS chromatograms of LB1 extracts in the positive and negative ESI mode.
mode. Since no authentic standard was available for the mass confirmation, seventeen components were identified based on the known structures and fragment ion patterns of previous documents (Table 2). These include catechin (Park et al., 2010), epigallocatechin (Xie et al., 2014), apigenin 6-C-glucosyl-8-C-arabinoside or apigenin 6-C-arabinosyl-8-C-glucoside (Kim et al., 2012), eriodictyol-O-glucoside (de Beer et al., 2012), luteolin-7-glucoside (Wojakowska et al., 2013), daidzein (Yoo et al., 2015), kaempferol (Yoo et al., 2015), naringenin (Wojakowska et al., 2013), genistein (Wojakowska et al., 2013), quercetin (Yoo et al., 2015), lespemyrtin F1 (Kim et al., 2012), 7,4′-dihydroxy-2′-methoxy-6-geranylisoflavone (Maximov et al., 2004), 2′,4′-dihydroxy-6″-methyl-6″-(4″-methylenpent-3-enyl)pyran(3″,2″,6,7)-isoflavanone (Maximov et al., 2004), lespedezol A6 (Kim et al., 2015b; Yoo et al., 2015), liquiritin (Yan et al., 2013), isoliquiritigenin (Yan et al., 2013), and lespemyrtin D1 (Kim et al., 2012).

The accurate mass and fragment ions of compounds 1–6, 8, 9, 11–13 and 15–17 were consistent with those from previous reports and mass bank (de Beer et al., 2012; Kim et al., 2015b; 2012; Maximov et al., 2004; Park et al., 2010; Wojakowska et al., 2013; Xie et al., 2014; Yan et al., 2013; Yoo et al., 2015). Compounds 7, 10 and 14 were tentatively characterized by the reference data. Among them, the levels of genistein, daidzein, quercetin and naringenin of LB1 were identified using
HPLC with reference standards (Fig. 6B). Concentration levels of genistein, daidzein, quercetin and naringenin in LB1 extracts are shown in (Table 3).

**Effects of LB1 components on MGO-induced glucotoxicity and cytotoxicity**

We examined the effect of LB1 components, such as genistein, daidzein, quercetin, and naringenin on glucotoxicity by AGEs formation assay. Fig. 7A shows AGEs were increased with BSA-MGO incubation, but AGEs were reduced after treating with genistein, quercetin, and naringenin.

TNBS assay was used to investigate whether LB1 components had the ability to break the preformed AGEs. Although genistein and quercetin increased the percentage of free amines restored from MGO-BSA after 24 h in a dose-dependent manner (Fig. 7B and Fig. S1B), daidzein and naringenin did not show any significant effects on the AGEs-breaking ability. This result suggests that genistein and quercetin had the AGEs-breaking activity which may contribute to the glucotoxicity reduction by LB1.

An MTT assay was also used to test the effects of four LB1 components on the cell viability of HUVECs. Although 1 mM AG reversed the MGO-induced reduction of HUVEC cell viability, it was not affected by...
pretreatment with these four LB1 components at any concentration (Fig. 7C and Fig. S1C). Taken together, our data suggest that these isoflavone constituents might not fully support the antiapoptotic function of LB1 extract.

Discussion

Lespedeza species have been used therapeutically as traditional medicine with antioxidant and anti-diabetic effects (Kim et al., 2015b). Among these species, Lespedeza Bicolor has been reported to have the most potent anti-inflammatory and antioxidant activities (Lee et al., 2016), and was previously used to treat nephritis, azotemia, and diuresis (Maximov et al., 2004). Therefore, we tested the 26 stalk samples of Lespedeza species for the protective effect against MGO-induced endothelial dysfunction by using cell-based screening assay, and found that LB1 was the most potent Lespedeza species acting on MGO-induced glucotoxicity without any cytotoxicity (Table 1 and Table S1). These difference in effectiveness is likely due to plants biodiversity. Through MTT assay, we confirmed protection effects of LB1 against MGO-induced cytotoxicity in a dose-dependent manner (Fig. 1).

LB1 also had protective effects in both the early and late stages of apoptosis. The protective effects of LB1 on cellular death was similar to that of aminoguanidine as judged by MTT assay (Fig. 1), and by an Annexin V-FITC/PI double labeling apoptosis assay (Fig. 2). Apoptosis in endothelial cells has been associated with several diseases related to diabetes and atherosclerosis (Rask-Madsen and King, 2013). Protection of endothelial cells from apoptotic cell death might be one of the
molecular mechanisms for LB1-mediated anti-diabetic and anti-atherosclerotic effects.

Bax and Bcl-2 are members of the Bcl-2 family which regulates cell apoptosis. p53 protein is known to regulate mitochondrial function and oxidative stress (Hori et al., 2013). p53 can also translocate to mitochondria and directly activate Bax (Reshi et al., 2016). Therefore, we first examined the expression of these apoptosis-related proteins in MGO treated HUVECs. Our results supported that pretreatment with LB1 ameliorates MGO-induced apoptosis by decreasing the expression of Bax and p53, and by increasing the expression of Bcl-2 (Fig. 3A–D).

MGO-induced cytotoxicity in endothelial cells is related to the activation of the MAPK signaling (Do et al., 2015). In this study, we observed that pretreatment with LB1 inhibited the MGO-induced MAPKs activation (Fig. 3E–H). These results demonstrate that LB1 has an inhibitory effect on MGO-induced apoptosis by inhibition of MAPKs activation in HUVECs.

Intracellular ROS generation in endothelial cells is also related to MGO treatment (Miyazawa et al., 2010). We confirmed that LB1 reduced MGO-induced ROS generation, as judged by the DCF-DA fluorescent dye staining method. Previously, it has been shown that the
Fig. 5. The effect of LB1 on MGO-induced glucotoxicity. A. The effects of LB1 on in vitro AGEs formation was examined by AGEs formation assay. BSA (5 mg/mL) was incubated with 2 mM MGO in the presence or absence of each sample in PBS for 7 days. B. AGEs-breaking ability of LB1 was evaluated by breaking of MGO-BSA using the TNBS assay. Baseline frequency of BSA free amines is represented by the dotted line at 100%. The percentage of each experiment is presented as the mean ± SD of three independent experiments. (**p < .001 vs. control and ###p < .001 vs. MGO treatment only).

Fig. 6. Chromatograms obtained from the LB1 extract. A. HPLC-Q-TOF-MS/MS chromatograms. a, total ion chromatogram (TIC) in the positive ion mode; b, TIC in the negative mode. 1, catechin; 2, epigallocatechin; 3, apigenin 6-C-glucosyl-8-C-arabinoside or apigenin 6-C-arabinosyl-8-C-glucoside; 4, eriodictyol-O-glucoside; 5, luteolin-7-glucoside; 6, daidzein; 7, kaempferol; 8, naringenin; 9, genistein; 10, quercetin; 11, lespcyrtin F1; 12, 7,4′-dihydroxy-2′-methoxy-6-geranylisoflavanone; 13, 2′,4′-dihydroxy-6′-methyl-6″-(4″-methylpent-3-enyl)pyranopyran (3″,2″,5,7)-isoflavanone; 14, lespedezol A6; 15, liquiritin; 16, isoliquiritigenin; 17, lespcyrin D1. B. HPLC chromatograms. a, chromatogram of standard compounds; b, chromatogram of LB1 extract.
MAPK signaling pathway is related to MGO-induced ROS generation (Heimfarth et al., 2013). In addition, intracellular ROS generation is caused by increased Bax protein expression (Pang et al., 2016). Thus, we propose here that LB1 might protect MGO-treated HUVECs from apoptosis via modulation of the MAPK signaling pathways and Bcl-2/Bax expression, and subsequent ROS generation.

Recent studies have shown that glycation might be regulated by various flavonoids. It also might show its structural differential effect with its inhibitory activities against AGEs formation (Xie and Chen, 2013). It was found that the hydroxylation on both A ring and B ring improved the inhibitory activity of AGEs formation. In this study, the inhibitory effect of LB1 and its components was evaluated as previously reported by Mesías et al. and Li et al. with slight modifications (Li et al., 2014; Mesías et al., 2013). LB1 stalk extract significantly inhibited AGEs formation in a dose-dependent manner in vitro (Fig. 5A). We confirmed the anti-glucotoxicity effect of the LB1 stalk extract using Table 2.

<table>
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<tr>
<th>No.</th>
<th>Tentative identification</th>
<th>tR</th>
<th>Measured mass(m/z)</th>
<th>Error (ppm)</th>
<th>Molecular formula</th>
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<td>Apigenin 6-C-glucosyl-8-C-arabinoside or apigenin 6-C-arabinosyl-8-C-glucoside</td>
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<tr>
<td>12</td>
<td>7,4'-Dihydroxy-2'-methoxy-6-geranylisoflavone</td>
<td>18.229</td>
<td>423.2131</td>
<td>6.86</td>
<td>C26H30O5</td>
<td>299.0888 (Maximov et al., 2004a)</td>
</tr>
<tr>
<td>13</td>
<td>2',4'-Dihydroxy-6'-methyl-6''-(4''-methylpent-3-enyl)pyranor (3',2'',6,7)-isoflavone</td>
<td>18.509</td>
<td>407.1852</td>
<td>−0.98</td>
<td>C25H26O5</td>
<td>274.1491 (Maximov et al., 2004a)</td>
</tr>
<tr>
<td>14</td>
<td>Lespedezol A6</td>
<td>18.689</td>
<td>421.1649</td>
<td>−0.74</td>
<td>C25H24O6</td>
<td>N.D (Kim et al., 2012)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>$r^2$</th>
<th>Concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>0.9997</td>
<td>0.053 ± 0.001</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.9999</td>
<td>0.165 ± 0.007</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.9994</td>
<td>0.853 ± 0.004</td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.9999</td>
<td>0.087 ± 0.001</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation.

Table 3.

Contents of four main components from LB1.

Fig. 7. The effect of LB1 components on MGO-induced cell viability and glucotoxicity. A. The effects of LB1 components on AGEs formation. B. The AGs-breaking ability of these LB1 components was evaluated by breaking of MGO-BSA using the TNBS assay. C. The effects of LB1 components on MGO-induced cell death in HUVECs. HUVECs were pretreated with four LB1 components, including genistein, daidzein, quercetin, and naringenin (10 μM each) for 1 h and then treated with 400 μM MGO for 24 h. Cell viability was analyzed by MTT assay. The percentage of each experiment is presented as the mean ± SD of three independent experiments. (**p < .001 vs. control and ###p < .001 vs. MGO treatment only).
endothelial dysfunction by reducing oxidative stress, MAPK activation, and ameliorating apoptotic cell death. Therefore, Lespedeza bicolor extract can be used as a potential supplement for prevention or treatment of MGO-induced endothelial dysfunction. Further studies are needed to identify the most potential chemical metabolite or combination of metabolites that are responsible for its anti-glucotoxicity effects.

Conflict of interest
We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary materials
Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2017.09.005.

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