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Chemical Components from the Stems of *Pueraria lobata* and Their Tyrosinase Inhibitory Activity

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Abstract – Phytochemical investigation of the stems of *Pueraria lobata* (Wild) Ohwi (Leguminosae), led to the isolation of eighteen known compounds: β-amyrone (**1**), (+)-pinoresinol (**2**), (+)-syringaresinol (**3**) (+)-syringaresinol-O-*β*-D-glucoside (**4**), (+)-lariciresinol (**5**), (−)-tuberosin (**6**), naringenin (**7**), liquiritigenin (**8**), isoliquiritigenin (**9**) genistein (**10**), daidzein (**11**) daidzin (**12**) daidzein 4',7-diglucoside (**13**) 2,4,4'-trihydroxy deoxybenzoin (**14**), S-(+)-1-hydroxy-3-(4-hydroxyphenyl)-1-(4-hydroxy-2-methoxy-phenyl)propan-2-one (**15**), methyl 2-O-*β*-D-glucopyranosylbenzoate (**16**), pyromeconic acid 3-O-*β*-D-glucopyranoside 6'- (O-4"-hydroxy-3-methoxybenzoate) (**17**), and allantion (**18**). The chemical structures of these compounds were elucidated from spectroscopic data and by comparison of those data with previously published results. The effects of isolated compounds on mushroom tyrosinase enzymatic activity were screened. The results indicated that, chloroform extract of *P. lobata* stems turned out to be having tyrosinase inhibitory effect, and only compounds **5**, **8**, **9**, and **11** showed enzyme inhibitory activity, with IC_{50} values of 21.49 ± 4.44, 25.24 ± 6.79, 4.85 ± 2.29, and 17.50 ± 1.29 μM, respectively, in comparison with these of positive control, kojic acid (IC_{50} 12.28 ± 2.72 μM). The results suggest that *P. lobata* stems extract as well as its chemical components may represent as potential candidates for tyrosinase inhibitors.
Keywords – *Pueraria lobata*, Leguminosae, Tyrosinase, Enzymatic activity, Inhibition

Introduction

Tyrosinase, also known as polyphenol oxidase, is a copper-containing enzyme that is widely distributed in microorganisms, animals, and plants.[1] Mushroom tyrosinase is particularly popular because it is readily available and useful for a number of applications. Tyrosinase inhibitors have generated considerable interest because of the key role of tyrosinase in mammalian melanogenesis and the enzymatic browning of fruit and fungi.[2] They are widely used in dermatological treatments and as ingredients in various cosmetics. Therefore, the development of safe and effective tyrosinase inhibitors has become an important goal for improving food quality and preventing pigmentation disorders and other melanin-related human health issues.[3] Plants are a rich source of bioactive chemicals that are mostly free from harmful side effects, and interest in finding tyrosinase inhibitors in such natural, bioactive materials is increasing.[1,2]

Pueraria lobata (Leguminosae; commonly known as kudzu) is widely distributed in temperate regions of far eastern Asia, including Korea, Japan, China, and India. Its root and flower are one of the earliest and most important plants used in traditional oriental medicine.[3] The roots are a common ingredient in traditional muscle relaxants, antipyretics, and treatments for cardiovascular diseases; the flowers are used in antitoxin treatments and to treat hypertension and alcoholism; and the stems have been used in medicines for malignant boils and acute pharyngitis.[4,5] Phytochemical studies on the roots of *P. lobata* have identified isoflavonoids, triterpenoids, polyphenols, and coumarins.[5,6] Although *P. lobata* is considered a noxious plant due to its high reproductive rate and vitality, the chemical constituents of the stem and its biological activities are worth studying.

In this study, we investigated the chemical constituents of *P. lobata* stems and evaluated their biological activities. We isolated compounds **1 - 18** (Fig. 1). This report details the isolation and structural determination of these compounds, and describes their effects on the enzymatic activity of mushroom tyrosinase. Dried stem parts were extracted with 100% methanol at room temperature. The crude extract was suspended in water and then successively partitioned with *n*-hexane, chloroform ($CHCl_3$), ethyl acetate

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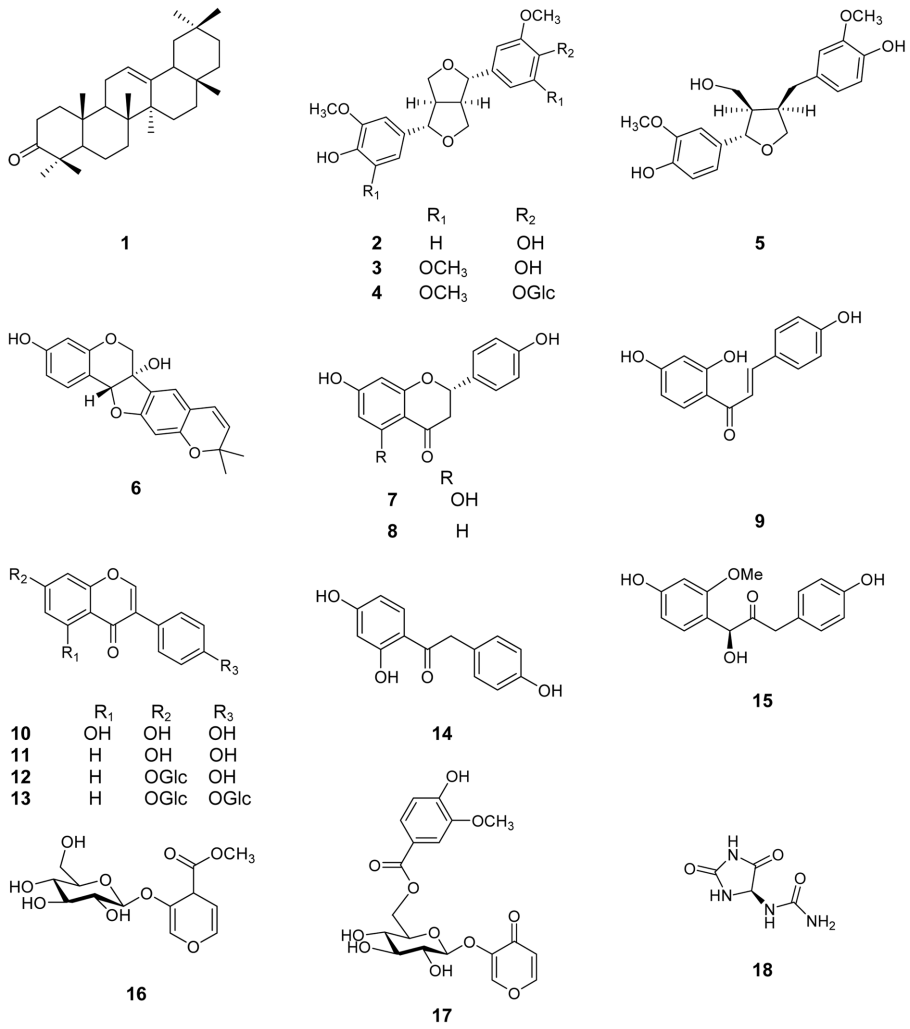

Fig. 1. Chemical structures of isolated compounds (**1 - 18**).

(EtOAc), and *n*-butanol (*n*-BuOH) to give *n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH, and water extracts, respectively, after removal of the solvents *in vacuo*. Flavonoids, triterpenoid, lignans, and alkaloids were isolated using various types of column chromatography. The isolated compounds were β-amyrone (**1**),[7] (+)-pinoresinol (**2**),[8] (+)-syringaresinol (**3**),[9] (+)-syringaresinol-O-*β*-D-glucoside (**4**),[10] (+)-lariciresinol (**5**),[11] (−)-tuberosin (**6**),[12] naringenin (**7**),[13] liquiritigenin (**8**),[14] isoliquiritigenin (**9**),[15] genistein (**10**),[16] daidzein (**11**),[17] daidzin (**12**),[18] daidzein 4',7-diglucoside (**13**),[19] 2,4,4'-trihydroxy deoxybenzoin (**14**),[20] S-(+)-1-hydroxy-3-(4-hydroxyphenyl)-1-(4-hydroxy-2-methoxyphenyl)propan-2-one (**15**),[21] methyl 2-O-*β*-D-glucopyranosylbenzoate (**16**),[22] pyromeconic acid 3-O-*β*-D-glucopyranoside 6'-(O-4"-hydroxy-3-methoxybenzoate) (**17**),[23] and allantion (**18**).[24] Their chemical structures (Fig. 1) were elucidated based on 1D and 2D NMR spectra, MS data, and by comparisons with previously published data acquired from similar compounds.

Experimental

Plant Material – Dried stems of *P. lobata* (Wild) Ohwi were collected in Okgye-myeon, Gangneung-si, Gangwon-do, Korea, in September 2012, and were identified by one of the authors, Young Ho Kim. A voucher specimen (CNU12109) was deposited at the herbarium, College of Pharmacy, Chungnam National University.

Extraction and isolation – The dried stems (2.9 kg) were thoroughly washed, cut into small pieces, and extracted three times (each for 8 h) with 9 L MeOH under reflux conditions. After removing the solvent *in vacuo*, the obtained residue (277.8 g) was dissolved in distilled water (1.0 L) to form a suspension that was successively

partitioned with *n*-hexane (2 L × 3), chloroform (2 L × 3), ethyl acetate (2 L × 3), and *n*-butanol (2 L × 3) to yield *n*-hexane-soluble (32.2 g), $CHCl_3$-soluble (12.7 g), EtOAc-soluble (44.9 g), *n*-BuOH-soluble (61.8 g), and aqueous (122.5 g) extracts, respectively, after removal of the solvents *in vacuo*. The $CHCl_3$ extract (12.0 g) was separated on a silica gel (70 - 230 mesh) column using a gradient elution of *n*-hexane and acetone (3:1–0:1 v/v) to afford five fractions (1a–1e). Repeated silica gel column chromatography of fractions 1a, 1b, and 1c was performed with gradient elutions of *n*-hexane and acetone (9:1–0:1 v/v) and *n*-hexane and EtOAc (70:1–0:1 v/v), followed by further separation on a YMC column eluted with MeOH and H_2O (1:1 and 0:1 v/v), yielding **1** (80.0 mg), **2** (5.0 mg), **3** (4.0 mg), **5** (14.0 mg), **6** (38.0 mg), and **9** (37.0 mg). The EtOAc extract was separated via column chromatography using silica gel (70–230 mesh) eluted with a gradient of $CHCl_3$ and acetone (6:1–1:1v/v) to give seven fractions (2a–2g). Repeated silica gel column chromatography of fractions 2a, 2b, and 2c with a gradient of *n*-hexane and acetone (5.5:1–0:1 v/v), and further separation on a YMC column eluted with MeOH and H_2O (1.5:1–0:1 v/v), yielded **7** (13.0 mg), **8** (15.0 mg), **10** (30.0 mg), **11** (10.0 mg), **14** (8.0 mg), and **15** (18.0 mg). Finally, the *n*-BuOH extract was separated on a silica gel (70–230 mesh) column eluted with a gradient of $CHCl_3$ and MeOH (9:1–0:1v/v) to give five fractions (3a–3e). Repeated silica gel column chromatography of fraction 3a with CH_2Cl_2 and MeOH (12:1–0:1 v/v), with further separation on a YMC column eluted with MeOH and H_2O (1:6–0:1 v/v) yielded **4** (5.0 mg), **16** (3.0 mg), and **17** (3.0 mg). Recrystallization (100% MeOH) of fractions 3b, 3c, and 3d yielded **12** (250.0 mg), **13** (480.0 mg), and **18** (566.0 mg), respectively.

β-Amyrone (**1**) – Orange gum; $C_{30}H_{48}O$; UV (MeOH) λ_{max} 206.0, 230.0, 324.0 nm; FT-IR (KBr) ν_{max} 3450, 2940, 1700, 1440, 1030 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[7] ESI-MS *m/z* 425.6 $[M+H]^+$.

(+)-Pinoresinol (**2**) – Light yellow needles; $C_{20}H_{22}O_6$; $[\alpha]_D^{20}$ +69 (*c* 0.1, MeOH); FT-IR (KBr) ν_{max} 3368, 2923, 2851, 1516, 1273 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[8] ESI-MS *m/z* 371.3 $[M+Na]^+$.

(+)-Syringaresinol (**3**) – Colorless needles; $C_{20}H_{26}O_8$; $[\alpha]_D^{20}$ +20 (*c* 0.1, MeOH); FT-IR (KBr) ν_{max} 3448, 2924, 1560, 1119 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[9] ESI-MS *m/z* 371.3 $[M+Na]^+$.

(+)-Syringaresinol-O-β-D-glucoside (**4**) – Colorless needles; $C_{28}H_{36}O_{13}$; UV (MeOH) λ_{max} 269.0, 243.0 nm; FT-IR (KBr) ν_{max} 3445, 2920,1565, 1121 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[10] ESI-MS *m/z* 603.5 $[M+Na]^+$.

(+)-Lariciresinol (**5**) – Amorphous liquid; $C_{20}H_{24}O_6$; $[\alpha]_D^{20}$ +30 (*c* 0.1, MeOH); FT-IR (KBr) ν_{max} 3275, 1516, 1030 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[11] ESI-MS *m/z* 383.4 $[M+Na]^+$.

(–)-Tuberosin (**6**) – Amorphous powder; $C_{20}H_{18}O_5$; $[\alpha]_D^{20}$ –130 (*c* 0.5, MeOH); FT-IR (KBr) ν_{max} 3044, 1618, 1112, 1027 cm^{-1}. UV (MeOH) λ_{max} 221.0, 285.0, 309.0 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[12] ESI-MS *m/z* 337.1 $[M-H]^-$.

Naringenin (**7**) – Orange needles; $C_{15}H_{12}O_5$; FT-IR (KBr) ν_{max} 3306, 2920, 1639, 1160 cm^{-1}. UV (MeOH) λ_{max} 223.0, 288.0 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[13] ESI-MS *m/z* 271.1 $[M-H]^-$.

Liquiritigenin (**8**) – Yellow amorphous powder; $C_{15}H_{12}O_4$; FT-IR (KBr): ν_{max} 3287, 2922, 1658, 1599, 1463, 1252 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[14] ESI-MS *m/z* 255.1 $[M-H]^-$.

Isoliquiritigenin (**9**) – Amorphous needles; $C_{15}H_{12}O_4$; FT-IR (KBr) ν_{max} 3125, 1630, 1514, 1228, 1024 cm^{-1}; UV (MeOH) λ_{max} 217.0, 243.0, 330 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[15] ESI-MS *m/z* 255.1 $[M-H]^-$.

Genistein (**10**) – Amorphous needles; $C_{15}H_{10}O_5$; FT-IR (KBr) ν_{max} 3191, 1654, 1621, 1577, 1516, 1287, 1252, 1176 cm^{-1}; UV (MeOH) λ_{max} 207.0, 260.0 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[16] ESI-MS *m/z* 269.0 $[M-H]^-$.

Daidzein (**11**) – Orange powder; $C_{16}H_{10}O_4$; FT-IR (KBr) ν_{max} 3124, 2920, 1624, 1572, 1241 cm^{-1}; UV (MeOH) λ_{max} 203.0, 247.0, 301.0 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[17] ESI-MS *m/z* 253.0 $[M-H]^-$.

Daidzin (**12**) – White powder; $C_{21}H_{20}O_9$; FT-IR (KBr) ν_{max} 3368, 2918, 1627, 1065 cm^{-1}; UV (MeOH) λ_{max} 201.0, 257.0 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[18] ESI-MS *m/z* 417.2 $[M+H]^+$.

Daidzein 4',7-diglucoside (**13**) – White amorphous powder; $C_{27}H_{30}O_{14}$; UV (MeOH) λ_{max} 201.0, 253.0 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[19] ESI-MS *m/z* 579.4 $[M+H]^+$.

2,4,4′-Trihydroxy deoxybenzoin (**14**) – Orange powder; $C_{14}H_{12}O_4$; FT-IR (KBr) ν_{max} 3285, 1675, 1613, 1299 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[20] ESI-MS *m/z* 273.1 $[M+H]^+$.

S-(+)-1-hydroxy-3-(4-hydroxyphenyl)-1-(4-hydroxy-2-methoxy-phenyl)propan-2-one (**15**) – Orange powder; $C_{16}H_{16}O_5$; $[\alpha]_D^{20}$ +202 (*c* 0.3, MeOH); FT-IR (KBr) ν_{max} 3330, 1715, 1613, 1513, 1198, 1031 cm^{-1}; ^1H and ^{13}C NMR data were in accordance with previously reported data;[21] ESI-MS *m/z* 289.1 $[M+H]^+$.

Methyl 2-O-β-D-glucopyranosyl-benzoate (**16**) – Colorless needles; $C_{14}H_{22}O_8$; ^1H and ^{13}C NMR data were in accordance with previously reported data;[22] ESI-MS *m/z* 319.3.1 $[M+H]^+$.

Pyromeconic acid 3-O-β-D-glucopyranoside 6'–(O-4"-hydroxy-3-methoxybenzoate) (**17**) – Yellow powder; $C_7H_{12}O_5$; ^1H and ^{13}C NMR data were in accordance with previously reported data;[23] ESI-MS *m/z* 175.1 $[M-H]^-$.

Allantion (**18**) – White powder; $C_4H_6N_4O_3$; FT-IR (KBr) ν_{max} 3337, 1725, 1670, 1028 cm^{-1}; UV (MeOH) λ_{max} 204.0, 228.0, 323 nm; ^1H and ^{13}C NMR data were in accordance with previously reported data;[24] ESI-MS *m/z* 157.0 $[M-H]^-$.

Tyrosinase Inhibitory Activity – Analyses of tyrosinase inhibitory activity were performed as previously described with slight modifications.[25,26] The reaction mixture, consisting of 80 μL 0.1 M sodium phosphate buffer (pH 6.8), 20 μL test sample dissolved in 100% methanol, 50 μL 2.0 mM L-tyrosine, and 50 μL mushroom tyrosinase (125 units/mL), was added to a 96-well plate. After incubation at 30 ºC for 20 min, the absorbance of each well was measured at 490 nm every 0 and 20 min with a spectrophotometer (Spectronic Genesys 6, Thermo Electron, Madison, WI). Kojic acid was used as a positive control.[27] The percent inhibition of tyrosinase activity was expressed using the following formula

$$\% \text{ inhibition rate } [(C_{20min} - C_{0min}) - (S_{20min} - S_{0min}) / (C_{20min} - C_{0min})] \times 100$$

where $C_{20\ min}$ and $C_{0\ min}$ are the absorbance of the control well after 20 and 0 min, and $S_{20\ min}$ and $S_{0\ min}$ are the absorbance of a sample well after 20 and 0 min, respectively.

Each assay was conducted in triplicate.

Statistical Analysis – Data were expressed as mean ± SD of experiments performed in triplicate. Statistical significance is indicated as determined by one-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 6 program (GraphPad Software Inc., San Diego, CA, U.S.A.), $p < 0.05$.

Result and Discussion

A methanolic extract of the dried stems of *P. lobata* was successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and BuOH. Repeated column chromatography, using silica gel and C-18 columns, of the $CHCl_3$-, EtOAc-, and BuOH-soluble fractions led to the isolation of 18 compounds (**1 - 18**) (Fig. 1). Combined analyses consisting of 1D and 2D NMR spectra, infrared absorbance spectra, and mass spectra were used to determine the chemical structures of these compounds. All of the physical and spectroscopic data were compared to those of previously published reports.

Structural elucidation of the isolated compounds – Compound **1**: The ^1H-NMR spectrum showed eight methyl singlet protons at δ_H 0.84 (3H, s, H-28), 0.88 (6H, s, H-29, 30), 1.02 (3H, s, H-26), 1.06 (3H, s, H-24), 1.07 (3H, s, H-25), 1.10 (3H, s, H-23), and 1.14 (3H, s, H-27), and one olefinic proton at δ_H 5.21 (1H, t, $J = 3.4$ Hz, H-12). The ^{13}C NMR spectrum exhibited 30 carbon resonances including those corresponding to 8 methyl carbon atoms at δ_C 15.1, 16.6, 21.4, 23.6, 25.8, 26.4, 26.8, and 32.1; 10 methylene carbon atoms at δ_C 19.6, 23.6, 26.0, 28.3, 32.4, 34.1, 34.6, 36.6, 39.2, and 46.7; 4 methine carbon atoms at δ_C 46.8, 47.4, 55.3, and 121.5; 7 quaternary carbon atoms at δ_C 39.7, 41.8, 47.2, 145.3, 31.0, 33.3, and 37.0; and 1 carbonyl group at δ_C 217.9. Based on the above spectral evidence, compound **1** was concluded to be β-amyrone. The physiochemical and spectral data were in good agreement with published data.[7] Compound **4**: The ^1H-NMR and ^{13}C NMR spectra indicated the presence of one glucose moiety, two benzene rings, two propane units, and four methoxyl groups in the molecule. These NMR data compared favorably with those published for (+)-syringaresinol-O-β-D-glucopyranoside.[10] Compound **5**: Peaks in the ^1H-NMR spectrum indicated the presence of two methoxyl group protons at δ_H 3.82 and 3.84, aromatic protons at δ_H 6.64 to 6.90, and furan ring protons at δ_H 2.37 and 4.74. The ^{13}C NMR spectrum exhibited 20 carbon resonances including those corresponding to 2 methoxyl groups at δ_C 56.4, 3 methylene carbon atoms at δ_C 33.7, 60.5, 73.6, aromatic carbon atoms between 110.8 to 149.2, and a furan ring at δ_C 44.0, 54.2, 73.6, and 84.2. Based on these data, compound **5** was concluded to be (+)-lariciresinol.[11] Compound **8**: The ^1H-NMR spectrum, contained ABX-type aromatic proton signals appearing at δ_H 7.73 (1H, d, $J = 8.7$ Hz, H-5), 6.45 (1H, dd, $J = 8.7$, 2.3 Hz, H-6), and 6.35 (1H, d, $J = 2.3$ Hz, H-8) due to an A-ring proton and A2B2-type aromatic proton signals at δ_H 7.32 (2H, dd, $J = 8.6$, 2.8 Hz) and 6.81 (2H, dd, $J = 8.6$, 2.9 Hz). The ^{13}C NMR spectrum exhibited 15 carbon resonances including those corresponding to 1 methylene carbon atom at δ_C 45.1; 8 tertiary carbon atoms at δ_C 81.2, 104.0, 111.9, 116.5, 116.5, 129.3, 129.3, and

130.1; 5 quaternary carbon atoms at δ_C 115.1, 131.5, 159.2, 165.8, 167.1; and 1 carbonyl carbon signal at δ_C 193.9. Based on these data, compound **8** was identified as liquiritigenin. These physiochemical and spectral data were in good agreement with previously published results.[14] Most of these compounds were isolated from the stem of *P. lobata* for the first time.

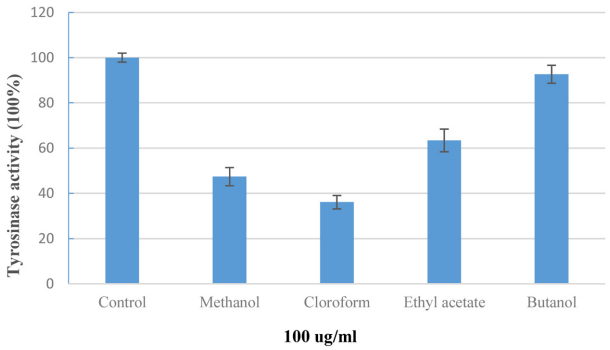

Fig. 2. Tyrosinase inhibitory activity of extracts obtained from stems of *P. lobata*.

Table 1. *In vitro* tyrosinase inhibitory activity of compounds **5, 8, 9** and **11**

Compound	IC_{50} [a] (mM)
(+)-lariciresinol **(5)**	21.49 ± 4.44
liquiritigenin **(8)**	25.24 ± 6.79
isoliquiritigenin **(9)**	4.85 ± 2.29
daidzein **(11)**	17.5 ± 1.29
Kojic acid[b]	12.27 ± 2.72

[a] All compounds were examined in a set of experiments three times.
[b] Positive control

Tyrosinase inhibitory activity – The effects of extracts (MeOH, $CHCl_3$, EtOAc, and BuOH) and the isolated compounds (**1 - 18**) on the enzymatic activity of mushroom tyrosinase were evaluated. Kojic acid, one of the most effective tyrosinase inhibitors, was used as a positive control (IC_{50}: 12.27 ± 2.72 μM). The results indicated that, in concentrations of 100 μg/mL, extracts (MeOH, $CHCl_3$, EtOAc, and BuOH) exhibited degrees of inhibition that were 52.6%, 63.9%, 36.6%, and 7.3% that of the control, respectively (Fig. 2). Among the isolated compounds, **5**, **8**, **9**, and **11** showed inhibition activities of more than 50% at concentrations of 20 μM and were therefore subject to further investigation (Fig. 3). The effects of those compounds were examined and the 50% inhibitory concentration (IC_{50}) was calculated using a dose-dependent response curve. The IC_{50} values of **5**, **8**, **9** and **11** were 21.49 ± 4.44, 25.24 ± 6.79, 4.85 ± 2.29 and 17.5 ± 1.29 μM, respectively. For comparison, the IC_{50} of the positive control, kojic acid, was 12.27 ± 2.72 μM (Table 1). To date, several phenolic compounds have been designed as potent tyrosinase inhibitors due to their structural similarities with the natural substrates L-tyrosine and L-DOPA.[25] There are few reports detailing the tyrosinase inhibitory effects of triterpenes and lignans.[2] Our results suggest that *P. lobata* extracts, and several of its chemical components, may be potential therapeutic tyrosinase inhibitors.

Acknowledgements

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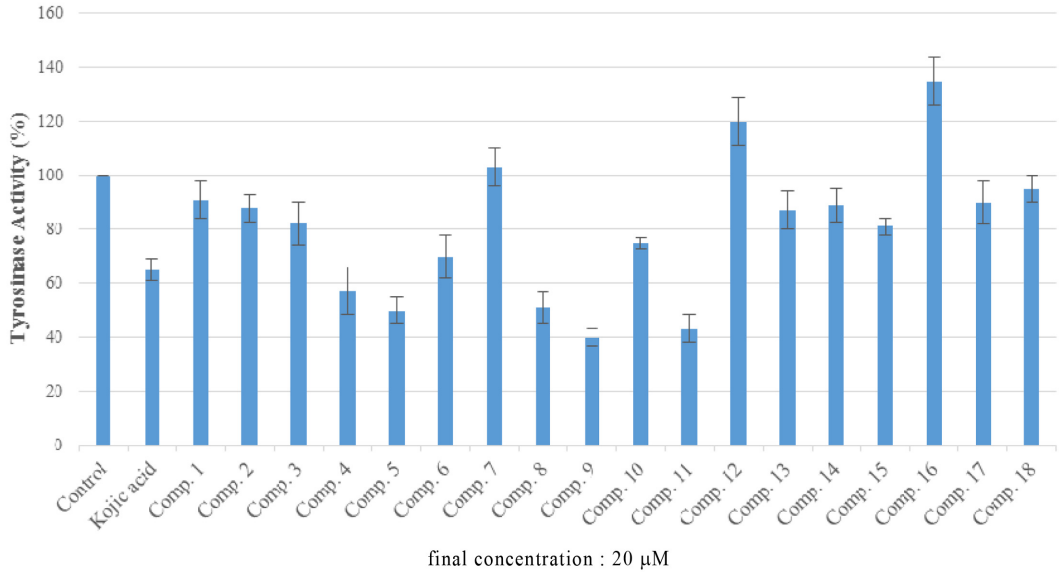

Fig. 3. Tyrosinase inhibitory activity of isolated compounds (**1 - 18**).

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