Natural Product Sciences
14(3) : 192-195 (2008)

Puerarol from the Roots of *Pueraria lobata* Inhibits the Formation of Advanced Glycation End Products (AGEs) *in vitro*

Jong Min Kim, Dae Sik Jang, Yun Mi Lee, Young Sook Kim, and Jin Sook Kim*

Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Daejeon 305-811, Korea

Abstract – Three known compounds, puerarol (**1**), pueroside B (**2**), and ononin (**3**), were isolated from an EtOAc-soluble fraction of the roots of *Pueraria lobata*. The isolates (**1** - **3**) were subjected to an *in vitro* bioassay to evaluate their inhibitory activity on the formation of advanced glycation end products (AGEs). Puerarol (**1**) exhibited a remarkable inhibitory activity on AGEs formation with IC_{50} value of 2.05 ± 0.32 μM as compared with positive control, aminoguanidine (IC_{50} value : 905.32 ± 7.58 μM).
Keywords – *Pueraria lobata*, Leguminosae, puerarol, advanced glycation end products (AGEs), diabetic complications

Introduction

The Diabetes Control and Complication Trial (DCCT) has identified hyperglycemia as the main risk-factor for the development of complications (DCCT research group, 1993). Hyperglycemia plays an important role in the pathogenesis of long-term complications, and diabetic patients with poor blood-glucose control are particularly at risk (Ahmed, 2005). Persistent hyperglycemia induces abnormal changes such as increase of advanced glycation end products (AGEs) formation (Makita *et al*., 1993), increase of polyol pathway (Shinohara *et al*., 1998), and activation of protein kinase C isomers (Larkins and Dunlop, 1992). AGEs are the final products of the nonenzymatic reaction between reducing sugars and amino groups in proteins, lipids, and nucleic acids (Singh *et al*., 2001). AGEs accumulation *in vivo* has been implicated as a major pathogenic process in diabetic complications, including neuropathy, nephropathy, and retinopathy (Ahmed, 2005). Thus, the discovery and investigation of AGEs inhibitors would offer a potential therapeutic approach for the prevention of diabetic or other pathogenic complications (Peng *et al*., 2008).

Pueraria lobata (Willd.) Ohwi [Leguminosae] is a deciduous woody vine and is widely distributed in temperate regions of Far Eastern Asia including Korea, Japan, Taiwan, NE China, and FE Russia. The root of *P. lobata* (Puerariae Radix) is an important Oriental medicine that has been widely used as an antipyretic, migraine, and antispasmodic agent (Bae, 2000). The constituents and bioactive substances of Puerariae Radix have been studied extensively (Kinjo *et al*., 1987; Ohshima *et al*., 1988; Lee *et al*., 1994).

Recently, puerariafuran and coumestrol has been isolated from the MeOH extract of the roots of *P. lobata* by our group as a potent inhibitor of AGEs formation *in vitro* (Jang *et al*., 2006). In the present study, three compounds (**1** - **3**) were further isolated from an EtOAc-soluble extract of the roots of *P. lobata* and they were evaluated for their inhibitory activity on the AGEs formation. The isolation and biological evaluation of the compounds **1** - **3** are described herein.

Experimental

Instruments and reagents – NMR experiments were conducted on a DRX-300 or Avance 500 FT-NMR (Bruker, Germany), and the chemical shifts were referenced to the residual solvent signals. TLC analysis was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness); compounds were visualized by dipping plates into 20% (v/v) H_2SO_4 reagent (Aldrich) and then heated at 110 °C for 5-10 min. Silica gel (Merck 60A, 70 - 230 or 230 - 400 mesh ASTM) and Sephadex LH-20 (Amersham Pharmacia Biotech) were used for column chromatography. All solvents used for the chromatographic separations were distilled before use.

Plant material – The roots of *Pueraria lobata* (Willd.)

*Author for correspondence
Fax: +82-42-868-9471; E-mail: jskim@kiom.re.kr

Ohwi were collected in Jeonmin-dong, Yuseong-gu, Daejeon, Korea, in March 2005 and indentified by Prof. Joo-Hwan Kim, Daejeon University, Daejeon 300-716, Korea. A voucher specimen (no. KIOM-K042) has been deposited at the Herbarium of the Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Korea.

Extraction and isolation – The air dried plant material (4.9 kg) was extracted with 20 L of MeOH three times by maceration. The extracts were combined and concentrated *in vacuo* at 40 °C to yield the MeOH extract (665 g). The concentrated extract (300 g) was suspended in H_2O (1.5 L) and then partitioned with *n*-hexane (3 × 1.5 L) to afford a *n*-hexane-soluble fraction (27 g) on drying. Next, the aqueous layer was partitioned again with EtOAc (3 × 1.5 L) to give an EtOAc-soluble fraction (24 g) and an aqueous residue. The EtOAc-soluble fraction (24 g) was chromatographed over silica gel (10 × 45 cm, 70 - 230 mesh) using a $CHCl_3$/MeOH gradient (from 1 : 0 → 0 : 1 v/v) to yield 12 fractions (F01 - F12). Fraction F07 [eluted with $CHCl_3$/MeOH (1 : 1 → v/v); 1.9 g] was further fractionated through a Sephadex LH-20 column chromatography (CC) [4 × 60 cm, MeOH-H_2O (4 : 1 v/v)] and a reversed phase silica gel CC [3 × 45 cm, MeOH-H_2O (2 : 3 v/v)] to afford puerarol (**1**, 9 mg). Fraction F08 [eluted with $CHCl_3$/MeOH (1 : 1 v/v); 1.1 g] was subjected to Sephadex LH-20 CC (3.5 × 60 cm, MeOH) to give ononin (**3**, 9 mg). Pueroside B (**2**, 50 mg) was purified from Fraction F12 [eluted with $CHCl_3$/MeOH (0 : 1 v/v); 4.2 g] by repeated CC.

Puerarol (1) – White powder; ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 7.67 (1H, d, J = 8.4 Hz, H-7), 7.59 (1H, s, H-1), 7.12 (1H, d, J = 1.8 Hz, H-10), 6.94 (1H, dd, J = 8.4, 2.1 Hz, H-8), 6.92 (1H, s, H-4), 5.35 (1H, m, H-13), 5.12 (1H, m, H-17), 3.31 (2H, d, J = 7.2 Hz, H-12), 2.05 (4H, m, H-15, 16), 1.69 (3H, s, H-19), 1.65 (3H, s, H-20), 1.57 (3H, s, H-21); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 159.4 (C-3), 158.9 (C-11a), 157.6 (C-6), 156.9 (C-10a), 155.9 (C-9), 152.8 (C-4a), 136.1 (C-14), 130.7 (C-18), 126.3 (C-1), 124.0 (C-2), 121.5 (C-17), 120.8 (C-7), 120.5 (C-13), 114.7 (C-1a), 113.8 (C-7a), 103.7 (C-8), 102.3 (C-6a), 101.9 (C-4), 98.5 (C-10), 39.0 (C-15), 27.2 (C-12), 26.0 (C-16), 25.4 (C-20), 17.5 (C-19), 15.8 (C-21)

Pueroside B (2) – White powder; ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 7.57 (1H, d, J = 8.0 Hz, H-6"), 6.95 (2H, d, J = 8.4 Hz, H-2', 6'), 6.90 (1H, d, J = 2.4 Hz, H-3"), 6.73 (1H, dd, J = 8.7, 2.4 Hz, H-5"), 6.63 (2H, d, J = 8.4 Hz, H-3', 5'), 6.33 (1H, d, J = 1.2 Hz, H-2), 6.03 (1H, m, H-4), 5.01 (1H, d, J = 7.0 Hz, glucosyl H-1), 3.84 (3H, s, OCH_3), 3.06 (1H, d, J = 3.4 Hz, H-4aα), 2.55 (1H, dd, J = 7.0, 14.0 Hz, H-4aβ); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 172.5 (C-1), 166.0 (C-3), 162.9 (C-4"), 156.3 (C-2"), 156.0 (C-4'), 131.3 (C-6"), 130.4 (C-2', 6'), 126.8 (C-1'), 114.8 (C-3', 5'), 113.4 (C-2), 112.1 (C-1"), 108.6 (C-5"), 101.2 (C-3"), 100.3 (glc-1), 83.6 (C-4), 77.4 (glc-5), 76.6 (glc-3), 73.1 (glc-2), 69.9 (glc-4), 60.8 (glc-6), 55.5 (OCH_3), 38.3 (C-4a)

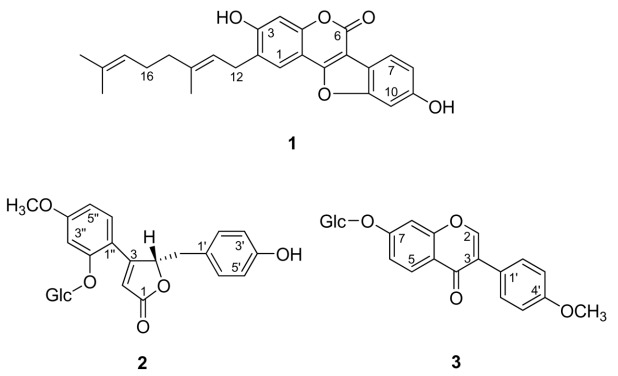

Fig. 1. Structures of compounds **1** - **3** from the roots of *Pueraria lobata*.

Ononin (3) – White powder; ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 8.43 (1H, s, H-2), 8.06 (1H, d, J = 9.0 Hz, H-5), 7.54 (2H, d, J = 8.7 Hz, H-2', 6'), 7.24 (1H, d, J = 2.4 Hz, H-8), 7.15 (1H, dd, J = 8.7, 2.4 Hz, H-6), 7.00 (2H, d, J = 8.7 Hz, H-3', 5'), 5.11 (1H, d, J = 7.2 Hz, glucosyl-H), 3.79 (3H, s, OCH_3); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 174.4 (C-4), 161.4 (C-7), 159.0 (C-4'), 157.0 (C-9), 153.6 (C-2), 130.0 (C-2', 6'), 126.9 (C-5), 124.0 (C-3), 123.3 (C-1'), 118.4 (C-10), 115.6 (C-6), 113.6 (C-3', 5'), 103.4 (C-8), 100.0 (glc-1), 77.2 (glc-3), 76.5 (glc-5), 73.1 (glc-2), 69.6 (glc-4), 60.6 (glc-6), 55.1 (OCH_3)

Determination of AGEs formation – According to the established method for measuring the formation of AGEs (Vinson and Howard, 1996), 10 mg/mL of bovine serum albumin (Sigma, St. Louis, MO, U.S.A.) in 50 mM phosphate buffer (pH 7.4), with 0.02% sodium azide to prevent bacterial growth, was added to 0.2 M fructose and glucose. The reaction mixture was then mixed with the isolated compounds or aminoguanidine (Sigma, St. Louis, MO, U.S.A.). After incubating at 37 °C for 14 days, the fluorescent reaction products were assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350 nm, Em: 450 nm). The AGEs assay was performed in quadruplicate. The concentration that resulted in 50% inhibition of the activity (IC_{50}) was estimated for each test sample from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

Results and Discussion

Three known compounds, puerarol (**1**), pueroside B (**2**), and ononin (**3**), were isolated from the EtOAc-soluble fraction of the root of *Pueraria lobata* by chromatographic methods. Their structures were elucidated by interpretation of their spectral data including ^{1}H- and ^{13}C-NMR spectroscopy as well as by comparison of spectral data with those of literature values as the followings.

The ^{1}H-NMR spectrum of **1** showed a set of ABX-type signals [δ 7.67 (1H, d, J = 8.4 Hz, H-7), 7.12 (1H, d, J = 1.8 Hz, H-10) and 6.94 (1H, dd, J = 8.4, 2.1 Hz, H-8), the presence of a 1,2,4-tri-substituted benzene ring, and two aromatic singlet signals [δ 7.59 (1H, s, H-1) and 6.92 (1H, s, H-4)]. The presence of a geranyl group in compound **1** is also revealed by four allylic signals [δ 5.35 (1H, m, H-13), 5.12 (1H, m, H-17), 3.31 (2H, d, J = 7.2 Hz, H-12), and 2.05 (4H, m, H-15, 16)] and three allylic methyl singlet signals [δ 1.69 (3H, s, H-19), 1.65 (3H, s, H-20) and 1.57 (3H, s, H-21)] in the ^{1}H-NMR spectrum of **1**. The ^{13}C-NMR data with DEPT experiments of **1** showed five methin carbons (δ 126.3, 120.8, 103.7, 101.9, 98.5), twelve quaternary carbons (δ 159.4, 158.9, 157.6, 156.9, 155.9, 152.8, 136.1, 130.7, 124.0, 114.7, 113.8, 102.3), and three methyl carbons (δ 25.4, 17.5, 15.8). On the basis of the above results, it was proposed that compound **1** is a geranylated coumestrol. The position of the geranyl group at C-2 was determined from HMBC correlations between δ_H 3.31 (H-12) and δ_C 124.0 (C-2), 126.3 (C-1) and 159.4 (C-3). Therefore, compound **1** was identified to be puerarol (2-geranyl-3,9-dihydroxycoumestan) (Ohshima *et al*., 1988).

The ^{1}H-NMR spectrum of **2** showed a set of ABX-type signals [δ 6.90 (1H, d, J = 2.4 Hz, H-3"), 7.56 (1H, d, J = 8.0 Hz, H-5"), and 6.73 (1H, dd, J = 2.4, 8.7 Hz, H-6")], a set of ABMX-type signals [δ 3.06 (1H, d, J = 3.4 Hz, H-4aα), 2.55 (1H, dd, J = 7.0, 14.0 Hz, H-4aβ), 6.03 (1H, m, H-4), and 6.33 (1H, d, J = 1.2 Hz, H-2)], two *ortho*-coupled doublets signals [δ 6.95 (2H, d, J = 8.4 Hz, H-2', 6') and 6.63 (2H, d, J = 8.4 Hz, H-3', 5')], an aromatic methoxyl signal at δ 3.84 (3H, s), and an β-anomeric proton signal at δ 5.05 (1H, d, J = 7.0 Hz) indicating that **2** has a sugar unit with β-linkage. The ^{13}C-NMR spectrum of **2** exhibited a carbonyl carbon (δ 172.3), an olefinic carbon (δ 113.4), six aliphatic tertiary carbon bearing an oxygen atom (δ 83.6, 77.4, 76.6, 73.1, 69.9, 60.8), an aliphatic secondary carbon (δ 38.3), an anomeric carbon (δ 100.3), and a methoxyl group (δ 55.3) in addition to two benzene ring units. Based on the above observations, it was proposed that compound **2** has a 4-methylenebut-2-en-4-olide moiety, a *para*-di-subsituted benzene ring, a tri-substituted benzene ring, β-D-glucosyl group, and a methoxyl group. By careful analysis of these results and by comparison with those in the literature (Nohara *et al*., 1993), compound **2** was elucidated as pueroside B [3-(2-*O*-glucopyranosyl-4-methoxyphenoyl)-4-(4-hydroxybenzyl)-but-2-en-4-olide].

The ^{1}H-NMR spectrum of **3** showed a resonance for an isoflavone skeleton that exhibited a diagnostic vinylic singlet at δ 8.43 (1H, s, H-2). Two *ortho*-coupled doublets centered at δ 7.00 (2H, d, J = 8.7 Hz, H-3', 5') and 7.54 (2H, d, J = 8.7 Hz, H-2', 6') were assigned to the protons of a *para*-di-substituted benzene ring (B ring). A set of ABX-type signals [δ 8.06 (1H, d, J = 9.0 Hz, H-5), 7.24 (1H, d, J = 2.4 Hz, H-8), and 7.15 (1H, dd, J = 8.7, 2.4 Hz, H-6)], a methoxyl signal at δ 3.79 (3H, s), a β-anomeric doublet at δ 5.11 (1H, d, J = 7.2 Hz, glc-1), and other multiplet resonances at δ 3.19~3.80 (glucosyl-H) indicated that **3** is a formononetin-*O*-β-D-glucoside. Thus, compound **3** was inferred to be ononin (formononetin-7-*O*-β-D-glucoside) and it was confirmed by comparison of its spectral data with those reported in the literature (Han *et al*., 2006).

The isolates (**1** - **3**) were subjected to an *in vitro* bioassay to evaluate their inhibitory activity on the formation of AGEs. Among these, puerarol (**1**) showed a remarkable inhibitory activity on the formation of AGEs with observed IC_{50} value of 2.05 ± 0.32 μM, better than the well know glycation inhibitor, aminoguanidine (IC_{50} value of 905.32 ± 7.58 μM), while pueroside B (**2**) and ononin (**3**) did not inhibit AGEs formation. Aminoguanidine, a hydrazine-like small molecule, is the first AGEs inhibitor explored in clinical trials. However, the drug was not ultimately approved for commercial production because side effects were observed in phase III clinical trials in patients with diabetes (Bolton *et al*., 2004). A number of natural inhibitors of AGEs formation have been reported up to date and many of them are flavonoids (Matsuda *et al*., 2003). However, natural compounds with very potent inhibitory activity on AGEs formation *in vitro* as puerarol (**1**) obtained from this work are rare. Although puerarol (**1**) has been isolated from Puerariae Radix, there was no report on its biological activity.

Thus puerarol (**1**) seems to be worthy of additional biological tests to more fully evaluate their potential as therapeutic agents for diabetic complications and related disease.

Acknowledgements

This research was supported by a grant [L08010] from

Korea Institute of Oriental Medicine. NMR was performed by the Korea Basic Science Institute (KBSI).

References

Ahmed, N., Advanced glycation end products role in pathology of diabetic complications. *Diabetes Res. Clin. Pract.* **67**, 3-21 (2005).

Bae, K.H., *Medicinal Plants of Korea*, Kyo-Hak Publishing Co., Seoul, 2000.

Bolton, W.K., Cattrom, D.C., Williams, M.E., Adler, S.G., Appel, G.B, Cartwright, K. Foiles, P.G., Freedman, B.I., Raskin, P., Ratner, R.E., Spinowitz, B.S., Whittier, F.C., Wuerth, J.P., and ACTION I Investigator Group, Randomized trial of an inhibitor of formation of advanced glycation end products in diabetic nephropathy. *Am. J. Nephrol.* **24**, 32-40 (2004).

The Diabetes Control and Complication Trial Research Group, The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **329**, 977-986 (1993).

Han, T., Li, H., and Zhang, Q., New thiazinediones and other components from *Xanthium strumarium*. *Chem. Nat. Compd.* **42**, 567-570 (2006).

Jang, D.S., Kim, J.M., Lee, Y.M., Kim, Y.S., Kim, J.H., and Kim, J.S., Puerariafuran, a new inhibitor of advanced glycation end products (AGEs) isolated from the roots of *Pueraria lobata*. *Chem. Pharm. Bull.* **54**, 1315-1317 (2006).

Kinjo, J.E., Furusawa, J.I., Baba, J., Takeshita, T., Yamasaki, M., and Nohara, T., Studies on the constituents of *Pueraria lobota*. III. Isoflavonoids and related compounds in the roots and the voluble stems. *Chem. Pharm. Bull.* **35**, 4846-4850 (1987).

Larkins, R.G. and Dunlop, M.E., The link between hyperglycaemia and diabetic nephropathy. *Diabetologia* **35**, 499-504 (1992).

Lee, S.J., Baek, H.J., Lee, C.H., and Kim, H.P., Antiinflammatory activity of isoflavonoids from Pueraria Radix and biochanin A derivatives. *Arch. Pharm. Res.* **17**, 31-35 (1994).

Makita, Z., Radoff, S., Rayfield, E.J., Yang, Z.H., Skolnik, E., Delaney, V., Friedman, E.A., Cerami, A., and Vlassara, H.N., Advanced glycosylation end products in patients with diabetic nephropathy. *N. Eng. J. Med.* **325**, 836-842 (1993).

Matsuda, H., Wang, T., Managi, H., and Yoshikawa, M., Structural requirements of flavonoids for inhibition of protein glycation and radical scavenging activities. *Bioorg. Med. Chem.* **11**, 5317-5323 (2003).

Nohara, T., Kinjo, J., and Furusawa, J., But-2-enolides from *Pueraria lobata* and revised structures of puerosides A, B and sophoroside A. *Phytochemistry* **33**, 1207-1210 (1993).

Ohshima, Y., Okuyama, T., Takahashi, K., Takizawa, T., and Shibata, S., Isolation and high performance liquid chromatography (HPLC) of isoflavonoids from the Pueraria root. *Planta Med.* **54**, 250-254 (1988).

Peng, X., Cheng, K., and Ma, J., Cinnamon bark proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **56**, 1907-1911 (2008).

Shinohara, R., Mano, T., Nagasaka, A., Sawai, Y., Uchimura, K., Hayashi, R., Hayakawa, N., Nagata, M., Makino, M., Kakizawa, H., Itoh, Y., Nakai, A., and Itoh, M., Effects of thyroid hormone on the sorbitol pathway in streptozotocininduced diabetic rats. *Biochim. Biophys. Acta* **1425**, 577-586 (1998).

Singh, R., Barden, A., Mori, T., and Beilin, L., Advanced glycation end-products: a review. *Diabetologia* **44**, 129-146 (2001).

Vinson, J.A. and Howard III, T.B., Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. *J. Nutr. Biochem.* **7**, 659-663 (1996).

(Accept September 9, 2008)