

Anthraquinones from the Seeds of *Cassia tora* with Inhibitory Activity on Protein Glycation and Aldose Reductase

Dae Sik JANG, Ga Young LEE, Young Sook KIM, Yun Mi LEE, Chan-Sik KIM, Jeong Lim YOO, and Jin Sook KIM*

Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine; 461-24 Jeonmin-dong, Yuseong-gu, Daejeon 305-811, Korea. Received June 18, 2007; accepted August 3, 2007

Nine anthraquinones, aurantio-obtusin (1), chryso-obtusin (2), obtusin (3), chryso-obtusin-2-*O*- β -D-glucoside (4), physcion (5), emodin (6), chrysophanol (7), obtusifolin (8), and obtusifolin-2-*O*- β -D-glucoside (9), isolated from an EtOAc-soluble extract of the seeds of *Cassia tora*, were subjected to *in vitro* bioassays to evaluate their inhibitory activity against advanced glycation end products (AGEs) formation and rat lens aldose reductase (RLAR). Among the isolates, compounds 6 and 8 exhibited a significant inhibitory activity on AGEs formation with observed IC₅₀ values of 118 and 28.9 μ M, respectively, in an AGEs-bovine serum albumin (BSA) assay by specific fluorescence. Furthermore, compounds 6 and 8 inhibited AGEs-BSA formation more effectively than aminoguanidine, an AGEs inhibitor, by indirect AGEs-ELISA. *N*^ε-Carboxymethyllysine (CML)-BSA formation was also inhibited by compounds 6 and 8. Whereas compounds 1, 4, and 6 showed a significant inhibitory activity on RLAR with IC₅₀ values of 13.6, 8.8, and 15.9 μ M, respectively.

Key words *Cassia tora*; Leguminosae; anthraquinone; advanced glycation end product; aldose reductase

The Diabetes Control and Complication Trial (DCCT) has identified hyperglycemia as the main risk-factor for the development of complications.¹⁾ Persistent hyperglycemia induces abnormal changes such as the formation of advanced glycation end products (AGEs),²⁾ the increase of sorbitol through the polyol pathway,³⁾ the overactivation of protein kinase C isoforms due to the synthesis of diacylglycerol (DAG).⁴⁾ Direct evidence indicating the contribution of AGEs in the progression of diabetic complications in different lesions of the kidneys, the rat lens, and in atherosclerosis has been recently reported.^{5,6)} *N*^ε-Carboxymethyllysine (CML) is one of the best characterized compounds of AGEs and can be detected in tissue and serum proteins by specific antisera.⁷⁾ Aldose reductase (AR), the key enzyme in the polyol pathway, also has been demonstrated to play important roles in the pathogenesis of diabetic complications and cataract formation.⁸⁾ Thus, the design and discovery of inhibitors of AGEs formation or AR can offer a promising therapeutic approach for the prevention of diabetic or other pathogenic complications.^{9,10)}

In our ongoing project directed toward the discovery of preventive agents for diabetic complications from the herbal medicines,¹¹⁾ the seeds of *Cassia tora* was chosen for more detailed investigation, since the EtOAc-soluble fraction of a MeOH extract showed a significant *in vitro* inhibitory effect on AGEs. *Cassia tora* (Leguminosae) is widely distributed in tropical Asian countries. The seeds of *C. tora* are reputed in Oriental medicine as vision-improving, antiasthenic, aperient, and diuretic agents.¹²⁾ *C. tora* have shown to possess various biological and pharmacological activities including anti-hepatotoxic,¹³⁾ radical scavenging,¹⁴⁾ antiallergic,¹⁵⁾ antimutagenic,¹⁶⁾ antifungal,¹⁷⁾ and antimicrobial.¹⁸⁾ Previous phytochemical investigation on the seeds of *C. tora* have resulted in the isolation of several anthraquinone and naphthopyrone derivatives.^{13,16–20)} In the present study, further fractionation of the EtOAc-soluble extract of the seeds of *C. tora* led to the purification of nine anthraquinones (1–9). The structures of 1–9 were determined by spectroscopic data interpretation

and they were subjected to *in vitro* bioassays to evaluate their inhibitory activity against AGEs and RLAR.

MATERIALS AND METHODS

General Experimental Methods Melting points were measured on an IA9100 melting point apparatus (Barnstead International, U.S.A.) and were quoted uncorrected. LREI was recorded on an Autospec (Micromass, U.K.). NMR experiments were conducted on a DRX-300 FT-NMR (Bruker, Germany), and the chemical shifts were referenced to the residual solvent signals. TLC analysis was performed on Kieselgel 60 F₂₅₄ (Merck) plates (silica gel, 0.25 mm layer thickness); compounds were visualized by dipping plates into 10% (v/v) H₂SO₄ reagent (Aldrich) and then heat treated 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.

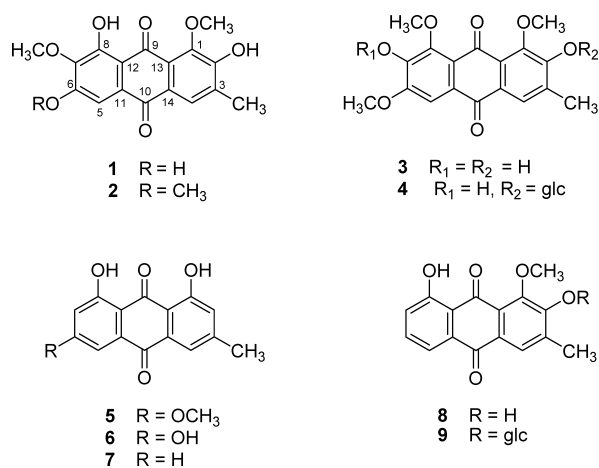


Fig. 1. Structures of Compounds 1–9 from the Seeds of *Cassia tora*

* To whom correspondence should be addressed. e-mail: jskim@kiom.re.kr

Plant Materials The seeds of *Cassia tora* L. (Leguminosae) were purchased from a commercial supplier in Goryung, Gyeongbuk, Korea, in January, 2005 and identified by Prof. J. H. Kim, Division of Life Science, Daejeon University, Daejeon 300—716. A voucher specimen (no. KIOM-H021) has been deposited at the Herbarium of the Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Korea.

Extraction and Isolation The ground seeds of *Cassia tora* (15 kg) were extracted three times with MeOH (60 l) at room temperature for 3 d. The combined solutions were then evaporated under reduced pressure at under 40 °C to give a MeOH extract (1302 g). The MeOH extract was suspended in H₂O (3 l) and successively extracted with *n*-hexane (3×3 l), EtOAc (3×3 l), and BuOH (3×3 l) to give *n*-hexane- (177 g), EtOAc- (95 g), BuOH- (172 g), and water-soluble fraction (858 g), respectively. Based on the initial biological testing, the EtOAc-soluble fraction (70.3 g, IC₅₀ value of 30.8 μg/ml against AGEs formation) was chromatographed over silica gel (φ 9×46 cm, 70—230 mesh) as the stationary phase using a CHCl₃–MeOH gradient (from 9:1→0:1 v/v) to yield 8 pooled fractions (fractions F01—F08). Of these, fractions F03 and F04 showed the most potent AGEs inhibitory activity (IC₅₀ value of <20 μg/ml). Fraction F03 [eluted with CHCl₃–MeOH (4:1 v/v), 20.6 g] was chromatographed over silica gel [φ 5×40 cm, 70—230 mesh, *n*-hexane–EtOAc (6:1→1:1) and CHCl₃–MeOH (6:1→0:1) gradient] to produce 7 subfractions (fractions F0301—F0307). Compounds **5** (96 mg) and **7** (353 mg) were obtained from fraction F0301 [eluted with *n*-hexane–EtOAc (6:1 v/v), 5.0 g] by a further silica gel column chromatography (φ 4×46 cm, 230—400 mesh, *n*-hexane–EtOAc gradient). Fraction F0305 [eluted with CHCl₃–MeOH (9:1 v/v), 2.9 g] was chromatographed over silica gel (φ 5×43 cm, 230—400 mesh, *n*-hexane–EtOAc gradient from 4:1 to 1:1 v/v) to give compounds **8** (932 mg), **6** (25.3 mg), **3** (302 mg), **2** (698 mg), and **1** (714 mg), in turn. Fraction F04 [eluted with CHCl₃–MeOH (4:1 v/v), 6.0 g] was purified over a further silica gel column (φ 6×44 cm, 230—400 mesh), with CHCl₃–MeOH (99:1→1:1) as solvent system, yielding two anthraquinone glucosides **4** (140 mg) and **9** (114 mg).

Chryso-obtusin-2-O-β-D-glucoside (4) Yellow powder; mp 229—230 °C; ESI-MS *m/z* (rel. int.): 521 [M+H]⁺; ¹H-NMR (DMSO-*d*₆, 300 MHz) δ 7.75 (1H, d, *J*=0.6 Hz, H-4), 7.48 (1H, s, H-5), 5.00 (1H, d, *J*=7.2 Hz, glucosyl H-1'), 3.98 (3H, s, OCH₃-6), 3.88 (3H, s, OCH₃-1), 3.870 (3H, s, OCH₃-7 or OCH₃-8), 3.867 (3H, s, OCH₃-7 or OCH₃-8), 3.60 (1H, m, glucosyl Ha-6), 3.42 (1H, m, glucosyl Hb-6), 3.00—3.28 (m, glucosyl H), 2.39 (1H, br s, CH₃-3); ¹³C-NMR (DMSO-*d*₆, 75 MHz) δ 181.2 (C-10), 180.7 (C-9), 156.6 (C-6), 154.3 (C-2), 153.3 (C-8), 151.8 (C-1), 147.7 (C-7), 139.2 (C-3), 129.4 (C-11), 128.8 (C-13), 127.3 (C-14), 124.1 (C-4), 122.9 (C-12), 105.4 (C-5), 103.6 (glucosyl C-1'), 77.3 (glucosyl C-5'), 76.4 (glucosyl C-3'), 74.0 (glucosyl C-2'), 69.8 (glucosyl C-4'), 61.7 (OCH₃-7 or OCH₃-8), 61.6 (OCH₃-7 or OCH₃-8), 60.9 (glucosyl C-6'/OCH₃-1), 56.3 (OCH₃-6), 17.4 (CH₃-3); Key HMBC correlations: H-4/C-2, C-3, C-10, C-14, CH₃-3; H-5/C-6, C-7, C-10, C-11, C-12; glucosyl H-1'/C-2; CH₃-3/C-2, C-3, C-4; OCH₃-6/C-6; OCH₃-7/C-7.

Determination of AGEs-BSA Formations by Fluorescence Spectroscopy (Fluorescent AGEs) According to the

method of Vinson and Howard,²¹⁾ the reaction mixture, 10 mg/ml of bovine serum albumin (BSA; 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 compounds or aminoguanidine (Sigma, St. Louis, MO, U.S.A.). After incubating at 37 °C for 14 d, the fluorescent reaction products were assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350 nm, Em: 450 nm).

Measurement of AGEs-BSA and CML-BSA Formations by Indirect ELISA After incubating, the aliquots were immediately frozen until their analysis by indirect enzyme-linked immunosorbent assay (ELISA). AGEs- and CML-ELISA were performed according to method of Engvall.²²⁾ Briefly, 96-well polystyrene plates were coated overnight with the reaction mixture (1.5 μg/ml) in 50 mM sodium carbonate buffer, pH 9.5—9.7. After coating, the wells were blocked for 1 h at 37 °C with PBS containing 1% BSA. Rabbit anti-AGEs antibodies (6D12, ThransGenic Inc., Kobe, Japan) or anti-CML antibodies (NF-1G, ThransGenic Inc., Kobe, Japan) were diluted at a titer of 1:3000 in PBS, incubated for 1.5 h at 37 °C, and washed. An alkaline phosphatase-conjugated antibody to rabbit IgG was then added as the secondary antibody at a titer of 1:3000 in PBS, incubated for 1 h at 37 °C, and washed again. The wells were developed with *p*-nitrophenyl phosphate substrate solution (pH 10.4). The reaction was terminated by adding 1 M sulfuric acid (50 μg/well) and the absorbance at 450 nm was read on a microplate reader (Synergy HT, BIO-TEK).

Measurement of RLAR Activity Rat lens was removed from the eyes of 8 weeks old Sprague-Dawley rats (Dae-Han Bio Link Co., Umsung, Korea) weighing 100—150 g and homogenized in 12 volumes of a 135 mM Na, K-phosphate buffer (pH 7.0) containing 0.5 mM phenylmethylsulfonyl fluoride and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 100000×*g* for 30 min, and the supernatant fluid was used as the crude rat lens aldose reductase (RLAR). RLAR activity was assayed according to the methods described previously^{23,24)} with slight modification. The incubation mixture contained 135 mM Na, K-phosphate buffer (pH 7.0), 100 mM lithium sulfate, 0.03 mM NADPH, 1 mM DL-glyceraldehyde as a substrate, and 50 μl of enzyme fraction, with or without 25 μl of sample solution, in a total volume of 1.0 ml. The reaction was initiated by the addition of NADPH at 37 °C and stopped by the addition of 0.3 ml of 0.5 M HCl. Then, 1 ml of 6 M NaOH containing 10 mM imidazole was added, and the solution was heated at 60 °C for 10 min to convert NADP to a fluorescent product. Fluorescence was measured using a spectrofluorometric detector (Shimadzu RF-5301PC, Japan, Ex: 360, Em: 460 nm).

Both AGEs and RLAR assays were performed in quadruplicate. The concentration of each test sample giving 50% inhibition of the activities (IC₅₀) was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

RESULTS AND DISCUSSION

Nine anthraquinones, aurantio-obtusin (**1**), chryso-obtusin (**2**), obtusin (**3**), chryso-obtusin-2-O-β-D-glucoside (**4**),

Table 1. Inhibitory Activity of Compounds from the Seeds of *C. tora* on Advanced Glycation End Products (AGEs) and Rat Lens Aldose Reductase (RLAR) *in Vitro*^{a)}

Compound	IC ₅₀ value (μM) ^{a)}	
	AGEs formation	RLAR
1	>1000	13.6
4	>1000	8.8
6	118	15.9
8	28.9	>100
AG ^{b)}	961	—
TMG ^{b)}	—	24.1

a) Inhibitory effect was expressed as mean of quadruplicate experiment. IC₅₀ values were calculated from the dose inhibition curve. b) AG (aminoguanidine) and TMG (3,3-tetramethyleneglutaric acid) were used as positive control. Compounds **2**, **3**, **5**, **7**, and **9** were not active in these bioassay systems.

physcion (**5**), emodin (**6**), chrysophanol (**7**), obtusifolin (**8**), and obtusifolin-2-*O*-β-D-glucoside (**9**) were isolated from an EtOAc-soluble extract of the seeds of *Cassia tora*. The structures of the known compounds were identified by physical and spectroscopic data (mp, ¹H-, ¹³C-NMR, and MS) measurement and by comparison with published values.^{25,26)} To the best of our knowledge, there are just two prior reports on the isolation of chryso-obtusin-2-*O*-β-D-glucoside (**4**)^{25,27)} and we now report the ¹³C-NMR data of **4** for the first time.

All the isolates **1**–**9** were subjected to *in vitro* bioassays to evaluate advanced glycation end products (AGEs) formation and rat lens aldose reductase (RLAR) inhibitory activity. The potential of **1**–**9** to inhibit AGEs-BSA formation is summarized in Table 1. A number of natural inhibitors of AGEs formation have been reported up to date, many of them are flavonoids with IC₅₀ values ranging from 90 to 200 μM.²⁸⁾ In this study, emodin (**6**) and obtusifolin (**8**) exhibited much stronger inhibitory activity on AGEs-BSA formation (IC₅₀ values of 118 and 28.9 μM, respectively) than aminoguanidine (IC₅₀ value of 961 μM), a well known glycation inhibitor, while others were found to be inactive. The inhibitory effects of emodin (**6**) and obtusifolin (**8**) on AGEs-BSA formation in the assay were reconfirmed by a specific AGEs-ELISA (absorbance of 450 nm). As shown in Fig. 2A, compounds **6** and **8** showed a dose-dependent inhibitory effect, the percentage inhibition of compounds **6** and **8** being more potent than that of aminoguanidine. CML-BSA formation was also inhibited by compounds **6** and **8** as a dose-dependent manner (Fig. 2B). AGEs adducts such as pyrraline, pentosidine and CML have been found at elevated levels in diabetics.²⁹⁾ AGEs inhibitors such as pyridoxamine and aminoguanidine prevent development of complications in experimental diabetes.³⁰⁾ We have previously reported that magnolol showed an *in vitro* inhibitory effect on the formation of AGEs and prevented the progression of diabetes and diabetic nephropathy in animal models of both type 1 and type 2 diabetes.³¹⁾ Emodin (**6**) was recently reported as a potent inhibitor of AGEs formation from the Rhei Rhizoma.³²⁾

In the RLAR inhibitory assay, emodin (**6**), which was active in the two protein glycation assay systems, also showed a good inhibitory activity on RLAR with IC₅₀ values of 15.9 μM. Aurantio-obtusin (**1**) and chryso-obtusin-2-*O*-β-D-glucoside (**4**) showed a significant inhibitory activity on RLAR with IC₅₀ values of 13.6 and 8.8 μM, respectively,

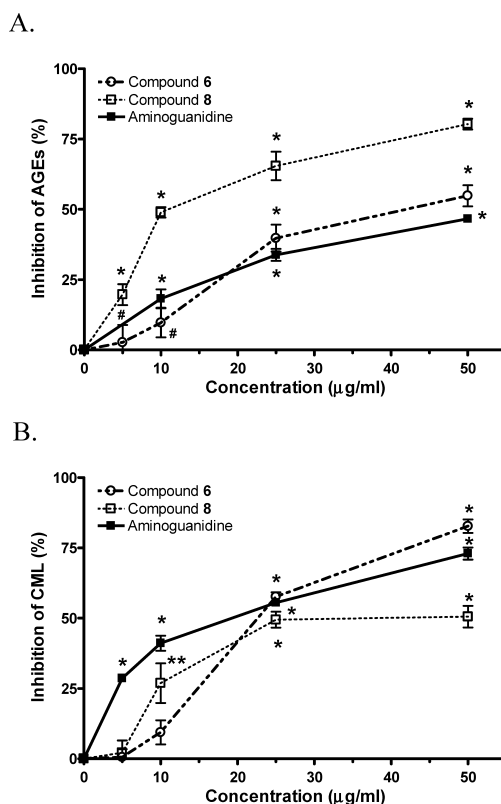


Fig. 2. Percentage Inhibition of Compounds **6** and **8** on AGEs (A) or CML-BSA (B) Formation Measured by ELISA with Rabbit Anti-AGEs Antibodies (6D12) or Anti-CML Antibodies (NF-1G): Inhibition by Different Concentrations of Compounds **6** (Emodin), **8** (Obtusifolin), and Aminoguanidine (AG)

Data are mean ± S.D. (n=3). *p<0.001, #p<0.01, **p<0.05 vs. incubated sample without compounds or AG, respectively.

which are comparable to that of tetramethyleneglutaric acid (IC₅₀ value of 24.1 μM), a positive control. Therefore, the dietary anthraquinones **1**, **4**, **6**, and **8** seem to be worthy of consideration as potential therapeutic agents for diabetic complications and related diseases through additional biological evaluation.

Acknowledgments We thank Prof. Joo-Hwan Kim, Division of Life Science, Daejeon University, Daejeon, Korea, for identifying the plant material. We also thank Korea Basic Science Institute (KBSI) for running NMR and MS experiments. This research was supported by a grant [L06010] from Korea Institute of Oriental Medicine.

REFERENCES

- 1) The Diabetes Control and Complication Trial Research Group, *N. Engl. J. Med.*, **329**, 977–986 (1993).
- 2) Makita Z., Radoff S., Rayfield E. J., Yang Z. H., Skolnik E., Delaney V., Friedman E. A., Cerami A., Vlassara H. N., *N. Eng. J. Med.*, **325**, 836–842 (1993).
- 3) Shinohara R., Mano T., Nagasaka A., Sawai Y., Uchimura K., Hayashi R., Hayakawa N., Nagata M., Makino M., Kakizawa H., Itoh Y., Nakai A., Itoh M., *Biochim. Biophys. Acta*, **1425**, 577–586 (1998).
- 4) Larkins R. G., Dunlop M. E., *Diabetologia*, **35**, 499–504 (1992).
- 5) Bucala R., Vlassara H., *Am. J. Kidney Dis.*, **26**, 875–888 (1995).
- 6) Kalousova M., Zima T., Tesar V., Stipek S., Sulkova S., *Kidney Blood Press Res.*, **27**, 18–28 (2004).
- 7) Reddy S., Bichler J., Wells-Knecht K. J., Thorpe S. R., Baynes J. W.,

- Biochemistry*, **34**, 10872—10878 (1995).
- 8) Beyer-Mears A., Cruz E., *Diabetes*, **34**, 15—21 (1985).
 - 9) Forbes J. M., Cooper M. E., Oldfield M. D., Thomas M. C., *J. Am. Soc. Nephrol.*, **14**, S254—S258 (2003).
 - 10) Yabe-Nishimura C., *Pharmacol. Rev.*, **50**, 21—33 (1998).
 - 11) Jang D. S., Kim J. M., Lee Y. M., Kim Y. S., Kim J.-H., Kim J. S., *Chem. Pharm. Bull.*, **54**, 1315—1317 (2006).
 - 12) Namba T., “Colored Illustrations of Wakan-Yaku,” Vol. 1, Hoi-kusha Publishing Co., Ltd., Osaka, 1998, p. 226.
 - 13) Wong S. M., Wong M. M., Seligmann O., Wagner H., *Planta Med.*, **55**, 276—280 (1989).
 - 14) Choi J. S., Lee H. J., Kang S. S., *Arch. Pharm. Res.*, **17**, 462—466 (1994).
 - 15) Zhang Z., Yu B., *J. Org. Chem.*, **68**, 6309—6313 (2003).
 - 16) Choi J. S., Lee H. J., Park K. Y., Jung G. O., Kang S. S., *Planta Med.*, **64**, 100—104 (1998).
 - 17) Kim Y. M., Lee C. H., Kim H. G., Lee H. S., *J. Agric. Food Chem.*, **52**, 6096—6100 (2004).
 - 18) Hatano T., Uebayashi H., Ito H., Shiota S., Tsuchiya T., Yoshida T., *Chem. Pharm. Bull.*, **47**, 1121—1127 (1999).
 - 19) Wong S. M., Wong M. M., Seligmann O., Wagner H., *Phytochemistry*, **28**, 211—214 (1989).
 - 20) Lee G. Y., Jang D. S., Lee Y. M., Kim J. M., Kim J. S., *Arch. Pharm. Res.*, **29**, 587—590 (2006).
 - 21) Vinson J. A., Howard III, T. B., *J. Nutr. Biochem.*, **7**, 659—663 (1996).
 - 22) Engvall E., *Methods Enzymol.*, **70**, 419—439 (1980).
 - 23) Kim H. Y., Oh J. H., *Biosci. Biotechnol. Biochem.*, **63**, 184—188 (1999).
 - 24) Matsuda H., Morikawa T., Toguchida I., Yoshikawa M., *Chem. Pharm. Bull.*, **50**, 788—795 (2002).
 - 25) Kitanaka S., Kitamura F., Tachio M., *Chem. Pharm. Bull.*, **33**, 1274—1276 (1985).
 - 26) Choi J. S., Jung G. H., Lee H. J., Kang S. S., *Arch. Pharm. Res.*, **19**, 302—306 (1996).
 - 27) Yun-Choi H. S., Kim J. H., Takido M., *J. Nat. Prod.*, **53**, 630—633 (1990).
 - 28) Matsuda H., Wang T., Managi H., Yoshikawa M., *Bioorg. Med. Chem.*, **11**, 5317—5323 (2003).
 - 29) Ahmed N., *Diabetes Res. Clin. Pract.*, **67**, 3—21 (2005).
 - 30) Stitt A., Gardiner T. A., Alderson N. L., Canning P., Frizzell N., Duffy N., Boyle C., Januszewski A. S., Chachich M., Baynes J. W., Thorpe S. R., *Diabetes*, **51**, 2826—2832 (2002).
 - 31) Sohn E. J., Kim C. S., Kim Y. S., Jung D. H., Jang D. S., Lee Y. M., Kim J. S., *Life Sci.*, **80**, 468—475 (2007).
 - 32) Nakagawa T., Yokozawa T., Kim Y. A., Kang K. S., Tanaka T., *Am. J. Chin. Med.*, **33**, 817—829 (2005).