Arch Pharm Res Vol 29, No 7, 587-590, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Naphthopyrone Glucosides from the Seeds of *Cassia tora* with Inhibitory Activity on Advanced Glycation End Products (AGEs) Formation

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(Received March 31, 2006)

Three naphthopyrone glucosides, cassiaside (**1**), rubrofusarin-6-O-β-D-gentiobioside (**2**), and toralactone-9-O-β-D-gentiobioside (**3**), were isolated from the BuOH-soluble extract of the seeds of *Cassia tora* as active constituents, using an *in vitro* bioassay based on the inhibition of advanced glycation end products (AGEs) to monitor chromatographic fractionation. The structures of **1-3** were determined by spectroscopic data interpretation, particularly by extensive 1D and 2D NMR studies. All the isolates (**1-3**) were evaluated for the inhibitory activity on AGEs formation *in vitro*.

Key words: *Cassia tora*, Leguminosae, Cassiaside, Rubrofusarin-6-*O*-β-D-gentiobioside, Toralactone-9-O-β-D-gentiobioside, 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). 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), activation of protein kinase C isomers (Larkins and Dunlop, 1992). Among them, enhanced formation and accumulation of AGEs are well known to be a major pathogenesis process in the normal aging as well as in the development of diabetes related complications (Bucala and Vlassara, 1995; Kalousova *et al.*, 2004). Thus, the design and discovery of inhibitors of the glycation cascade can offer a promising therapeutic approach for the prevention of diabetic or other pathogenic complications (Forbes *et al.*, 2003).

In our ongoing project directed toward the discovery of preventive agents for diabetic complications from the herbal medicines and prescriptions (Kim *et al.*, 2002), the seeds of *Cassia tora* was chosen for more detailed investigation, since the BuOH-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, asperient, and diuretic agents (Namba, 1980). *C. tora* have shown to possess various biological and pharmacological activities including antihepatotoxic (Wong *et al.*, 1989b), antiallergic (Zhang and Yu, 2003), antimutagenic (Choi *et al.*, 1998), antifungal (Kim *et al.*, 2004), radical scavenging (Choi *et al.*, 1994), and antimicrobial (Hatano *et al.*, 1999). Previous phytochemical investigation on the seeds of *C. tora* have resulted in the isolation of several anthraquinone, and naphthopyrone derivatives (Wong *et al.*, 1989a, 1989b; Choi *et al.*, 1998; Hatano *et al.*, 1999; Kim *et al.*, 2004). However, no pharmacological studies regarding AGEs inhibition have been carried out on the seeds of *C. tora* to date. In the present study, three naphthopyrone glucosides (**1-3**) were isolated from the BuOH-soluble extract of the seeds of *C. tora*, using an *in vitro* bioassay based on the inhibition of AGEs to monitor chromatographic fractionation. The structures of **1-3** were determined by spectroscopic data interpretation and they were subjected to an *in vitro* bioassay to evaluate AGEs inhibitory activities.

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MATERIALS AND METHODS

General experimental procedures

Melting points were measured on an IA9100 melting point apparatus (Barnstead International, U.S.A.) and were quoted uncorrected. LRESI was recorded on a Mariner mass spectrometer (Perspective Biosystem, U.S.A.). 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 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness); compound were visualized by dipping plates into 10% (v/v) H_2SO_4 reagent (Aldrich) and then heat treated at 110°C for 5-10 min. Silica gel (Merck 60A, 70-230 or 230-400 mesh ASTM), Sephadex LH-20 (Amersham Pharmacia Biotech), and reversed-phase silica gel (YMC Co., ODS-A 12 nm S-150, S-75 μm) were used for column chromatography. All solvents used for the chromatographic separations were distilled before use.

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* (6 kg) were extracted three times with MeOH (42 L) at room temperature for 2 days. The combined solutions were then evaporated under reduced pressure at under 40°C to give a MeOH extract (364.1 g). The MeOH extract was suspended in H_2O (2 L) and successively extracted with *n*-hexane (3×2 L), $CHCl_3$ (3×2 L), EtOAc (3×2 L), and BuOH (3×2 L) to give *n*-hexane- (58.5 g), $CHCl_3$- (51.0 g), EtOAc- (6.0 g), BuOH- (54.4 g), and water- soluble fraction (194.2 g), respectively. Based on the initial biological testing, the BuOH-soluble fraction (41.5 g) was chromatographed over silica gel (ϕ 10×70 cm, 70-230 mesh) as the stationary phase using a $CHCl_3$-MeOH gradient (from 9:1 → 0:1 v/v) to yield 10 pooled fractions (fractions F01-F10). Of these, fractions F04 and F05 showed the most potent AGEs inhibitory activity (IC_{50} value of < 20 μg/mL). Compound **1** (1.1 g) was obtained from fraction F04 [eluted with $CHCl_3$-MeOH (4:1 v/v), 2.58 g] by recrystallization in MeOH. Fraction F05 [eluted with $CHCl_3$-MeOH (4:1 v/v), 12.81 g] was chromatographed over silica gel (ϕ 7×80 cm, 70-230 mesh, $CHCl_3$-MeOH-H_2O gradient from 13:1:0 to 7:4:1 v/v) to produce 11 subfractions (fractions F0501-F0511). Compounds **2** (98 mg) and **3** (36 mg) were purified from fraction F0506 by reversed-phase column chromatography (ϕ 3.6×34 cm, S-150 μm; MeOH-H_2O=6:4 v/v).

Cassiaside (1)

Yellowish powder: mp 258-260°C; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 2.36 (3H, s, CH_3-2), 3.22-3.79 (m, glucosyl-H), 5.07 (1H, d, *J* = 7.8 Hz, glucosyl H-1), 6.14 (1H, s, H-3), 6.72 (1H, d, *J* = 2.0 Hz, H-9), 6.68 (1H, d, *J* = 2.0 Hz, H-7), 7.04 (1H, s, H-10), 10.3 (1H, s, OH-8), 14.9 (1H, s, OH-5); ^{13}C-NMR (75 MHz DMSO-d_6) see the Table I. LRESIMS *m/z* 421 ([M+H]$^+$).

Rubrofusarin-6-*O*-β-D-gentiobioside (2)

Yellowish powder, mp 184-186°C; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 2.39 (3H, s, CH_3-3), 3.88 (3H, s, OCH_3-8), 3.00-3.99 (m, glycosyl-H), 4.20 (1H, d, *J* = 7.8 Hz, glucosyl H-1), 5.07 (1H, d, *J* = 7.8 Hz, glucosyl H-1), 6.19 (1H, s, H-3), 6.80 (1H, d, *J* = 2.4 Hz, H-7), 6.93 (1H, d, *J* = 2.1 Hz, H-9), 7.19 (1H, s, H-10); ^{13}C-NMR (75 MHz DMSO-d_6) see the Table I. LRESIMS *m/z* 597 ([M+H]$^+$).

Toralactone-9-*O*-β-D-gentiobioside (3)

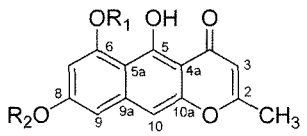
Yellowish powder, mp 220-222°C; ^{1}H-NMR (300 MHz, DMSO-d_6) ä: 2.22 (3H, s, -CH_3), 3.00-3.99 (m, glycosyl-H), 3.89 (3H, s, OCH_3-7), 4.20 (1H, d, *J* = 7.8 Hz, glucosyl H-1), 5.10 (1H, d, *J* = 7.8 Hz, glucosyl H-1), 6.50 (1H, d, *J* = 0.9 Hz, H-4), 6.87 (1H, d, *J* = 2.4 Hz, H-8), 6.92 (1H, d, *J* = 2.4 Hz, H-6), 7.13 (1H, s, H-5); ^{13}C-NMR (75 MHz DMSO-d_6) see the Table I. LRESIMS *m/z* 597 ([M+H]$^+$).

Determination of AGEs formation

According to well established method (Vinson and Howard, 1996), the reaction mixture, 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 compounds or aminiguanidine (Sigma, St Louis, MO, U.S.A.). After incubating at 37 for 7 days, the fluorescent reaction products were assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350, Em: 450 nm). AGEs assay was performed in quadruplicate. The concentration of each test sample giving 50% inhibition of the activities (IC_{50}) was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

RESULTS AND DISCUSSION

Three naphthopyrone glucosides, cassiaside (**1**) (Choi *et al.*, 1994; Wong *et al.*, 1989b), rubrofusarin-6-*O*-β-D-



1 R_1= glucosyl, R_2= H
2 R_1= gentiobiosyl, R_2= CH_3

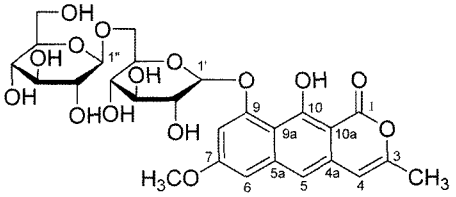


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Fig. 1. Structures of compounds **1-3** isolated from *Cassia tora*.

gentiobioside (**2**) (Choi *et al.*, 1994; Wong *et al.*, 1989b), and toralactone-9-*O*-β-D-gentiobioside (**3**) (Wong *et al.*, 1989b), were isolated from the BuOH-soluble extract of the seeds of *C. tora* as active constituents, using an *in vitro* bioassay based on the inhibition of AGEs to monitor chromatographic fractionation. Their structures were identified by physical and spectroscopic data (mp, MS, ^{1}H-, ^{13}C-, and 2D-NMR) measurement and by comparison with published values.

Table I. ^{13}C-NMR Spectral Data for Compounds **1-3** (in DMSO-d_6)

position	Compounds (δ_C, ppm)		
	1	**2**	**3**
1			162.8
2	168.6	168.7	
3	106.5	106.7	152.6
4	183.7	183.7	104.1
4a	103.0	101.1	132.4
5	162.1	161.9	111.6
5a	106.9	107.7	141.6
6	158.3	157.6	98.5
7	101.3	100.8	161.5
8	159.7	161.1	100.8
9	102.5	99.7	157.6
9a	140.5	140.3	109.2
10	100.0	103.6	166.8
10a	152.3	152.4	101.8
CH_3	20.2	20.1	18.8
OCH_3		55.5	55.5
Glc-1'	101.2	103.5	103.6
-2'	73.5	73.5	73.4
-3'	76.4	76.9	76.9
-4'	69.6	70.0	70.1
-5'	77.3	75.5	75.5
-6'	60.7	68.7	68.8
Glc-1"		99.7	100.8
-2"		73.5	73.5
-3"		76.3	76.4
-4"		69.6	69.6
-5"		76.6	76.7
-6"		61.0	61.0

The potential of compounds **1-3** to inhibit AGEs formation was summarized in Table II. In recent investigation, flavone and flavonol derivatives have been found to exhibit a significant *in vitro* inhibitory activity against AGEs formation (Matsuda *et al.*, 2003). In the present study, three naphthopyrone glucosides, cassiaside (**1**), rubrofusarin-6-*O*-β-D-gentiobioside (**2**), and toralactone-9-*O*-β-gentiobioside (**3**) (IC_{50} value; 32.2, 20.3, and 6.4 μg/mL, respectively), showed more potent *in vitro* inhibitory activity against AGEs formation than a positive control, aminoguanidine (IC_{50} value of 34.6 μg/mL). It was also observed that the diglucosides (**2** and **3**) were more efficient than the monoglucoside (**1**) in this assay system. However, further investigations are needed to determine the structure-activity relationship of naththopyrone derivatives regarding inhibition of AGEs formation. Although there are some reports on biological activities of cassiaside (**1**), rubrofusarin-6-*O*-β-D-gentiobioside (**2**), and toralactone-9-O-β-gentiobioside (**3**) (Wong *et al.*, 1989b; Choi *et al.*, 1994, 1997), this is the first report on inhibitory activity of these compounds against AGEs formation. Therefore, compounds

Table II. Inhibitory activity of compounds from the seeds of *Cassia tora* on AGEs formation *in vitro*[a]

Compound	Conc. (μg/mL)	Inhibition (%)	IC_{50} value [μg/mL (μM)]
1	10	25.4±0.7	32.2 (76.7)
	25	40.9±3.8	
	50	70.6±1.5	
2	10	38.5±1.5	20.3 (34.1)
	25	54.3±2.5	
	50	85.9±1.4	
3	2.5	9.3±3.2	6.4 (10.7)
	5	38.1±9.4	
	10	85.1±7.4	
Aminoguanidine (positive control)	18.5	25.3±3.2	34.6 (467)
	37	54.6±0.9	
	74	69.7±0.9	

[a]Inhibitory effect was expressed as mean±S.D. of quadruplicate experiments. IC_{50} values were calculated from the dose inhibition curve.

1-3 will be of candidates for additional biological evaluation to further define their potential as natural therapeutic agents for diabetic complications and related diseases.

ACKNOWLEDGEMENTS

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.

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