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Alaternin, Cassiaside and Rubrofusarin gentiobioside, Radical Scavenging Principles from the Seeds of *Cassia tora* on 1,1-Diphenyl-2-picrylhydrazyl(DPPH) Radical

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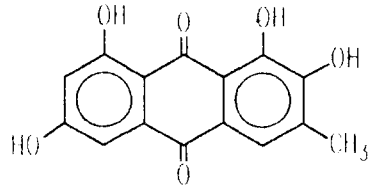
Radical scavenging principles on 1,1-diphenyl-2-picrylhydrazyl(DPPH) radical were isolated from the seeds of *Cassia tora* L.. Assignments of the ^{1}H- and ^{13}C-NMR data showed the active components to be an anthraquinone, alaternin and two naphthopyrone glycosides, nor-rubrofusarin-6-β-D-glucoside(cassiaside) and rubrofusarin-6- -D-gentiobioside. Alaternin showed more potent radical scavenging effect than the others.

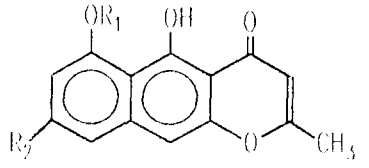
Key words: Anthraquinone, 1,1-Diphenyl-2-picrylhydrazyl, Radical scavenger, Naphthopyrone glycosides, *Cassia tora*

INTRODUCTION

In the search for pharmacologically active plant contituents, several species of Korean medicinal plants were evaluated, and a methanolic extract from the seeds of *Cassia tora* (Leguminosae) was found to have radical scavenging activity on DPPH radical(Choi *et al.*, 1993). The seeds of *Cassia tora* are used to improve visual activity in Chinese medicine and it is also reputed for its medicinal value as asperient, antiasthenic, and diuretic agents (Namba, 1980). In Korea, the hot aqueous extract of the seeds is taken orally for the protection of the liver (Yun and Chang, 1977). Antihepatotoxic naphthopyrone glycosides were isolated from this plant (Wong *et al.*, 1989). We now report the isolation and the structure elucidation of the active radical scavenging principles on the DPPH radical.



compound 7



compound 8 : R_1 = glucoside, R_2 = OH
compound 9 : R_1 = gentiobioside, R_2 = OCH_3

MATERIALS AND METHODS

Melting points were determined on a Electrothermal digital micro melting point apparatus and without correction. IR spectra were recorded on a Shimadzu IR-400 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were determined at 300 MHz and 75.5 MHz, respectively, on a Brucker AM 300 spectrometer with tetramethylsilane as the internal standard. Chemical shifts are given in δ (ppm) and multiplicities are indicated as follows: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br=broad. Coupling constants (J) are given in herts (Hz). Column chromatographies were performed on Kieselgel 60 (70-230 mesh; Merck). TLC was carried out with precoated Kieselgel 60 F_{254} plates (Merck) and spots were determined under UV irradiation and by heating on a hot plate after spraying 50% sulfuric acid reagent.

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Isolation

The powdered seeds of *C. tora* (3 kg) was extracted four times with methanol at 95℃ and the extract was concentrated *in vacuo*. The residue (900 g) was dissolved in water and successively extracted with dichloromethane, ethyl acetate and *n*-butanol. The dichloromethane extract was chromatographed on silica gel with mixtures of dichloromethane and methanol of increasing polarity, and divided 15 subfractions (Fr. 1 to Fr. 15). Subfraction 1 which was eluted with dichloromethane to give chrysophanol (**1**, 60 mg) and physcion (**2**, 250 mg). Subfraction 2 eluted with dichloromethane-methanol (30 : 1) to afford β-sitosterol (**3**, 500 mg) and chryso-obtusin (**4**, 150 mg). Subfraction 3 eluted with dichloromethane-methanol (20 : 1) to give emodin (5, 80 mg) and aurantio-obtusin (**6**, 100 mg). Subfraction 4 eluted with dichloromethane-methanol (15 : 1) to give active alaternin (**7**, 50 mg). The ethyl acetate extract (20 g) was chromatographed on silica gel with mixtures of ethyl acetate and methanol of increasing polarity, and divided 3 subfractions (Fr. 1 to Fr. 3). Subfraction 1 which was eluted with ethyl acetate gave nor-rubrofusarin-6-β-D-glucoside (**8**, 50 mg). The butanol extract (90 g) was chromatographed on silica gel with mixtures of ethyl acetate and methanol of increasing polarity, and divided 17 fractions(Fr. 1 to Fr. 17). Fractions 1 and 2 were combined and rechromatographed with ethylacetate to give nor-rubrofusarin-6-β-D-glucoside (**8**, 50 mg). Fraction 6 and 7 was combined and rechromatographed with ethyl acetate-MeOH-H_2O (300 : 35 : 10) to give rubrofusarin-6-β-D-gentiobioside (**9**, 50 mg) and chrysophanol-triglucoside (**10**, 40mg).

Alaternin (7)

Yellowish needles from methanol, mp 296-298℃.

Table I. ^{1}H-NMR chemical shifts for anthraquinones in DMSO-d_6

Compound Proton No.	emodin[a]	7-hydroxy emodin[b]	2-hydroxy emodin[c]	compound 7
H-1				
H-2	7.16	6.97		
H-3				
H-4	7.49	7.29	7.48	7.47
H-5	7.12	7.16	7.04	7.08
H-6				
H-7	6.59		6.48	6.52
H-8				
OH	12.08			12.02
OH	12.01			
OH	11.37			
CH_3	2.41	2.38	2.24	2.34

[a](Danielsen *et al.*, 1992). [b](Chan and Crow, 1966). [c](Rauwald and Just, 1981).

^{1}H- and ^{13}C-NMR: Table I and II

Cassiaside (8)

Yellowish powder from methanol, mp 257-259℃

IR (cm $^{-1}$, KBr): 3300(OH), 1630(conjugated C=O), 1615, 1580 (aromatic ring), ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 2.36 (3H, s, -CH_3), 3.10-5.05 (m, glucosyl-H), 6.12 (1H, s, H-3), 6.72 (2H, s, H-7 & H-9), 7.15 (1H, s, H-10), ^{1}H-NMR of the glucosyl protons after D_2O exchange, δ: 3.25-3.80 (5H, m, glucosyl H), 4.90 (1H, d, J=6.0Hz, anomeric H), ^{13}C-NMR (DMSO-d_6, 75.5 MHz) δ: 183.52, 168.38, 161.91, 159.56, 158.15, 152. 15, 140.30, 106.87, 106.36, 102.94, 102.50, 101.77, 101.43, 99.81, 77.17, 76.27, 73.47, 69.80, 69.60, 60.67, 19.95.

Rubrofusarin-6-β-D-gentiobioside (9)

Yellowish needles from methanol, mp 186-188℃

^{1}H-NMR (300 MHz, DMSO-d_6) δ: 2.38 (3H, s, -CH_3), 3.88 (3H, s, -OCH_3), 3.00-5.00 (sugar-H), 5.05 (1H, d, J=7.5 Hz, anomeric H), 5.06 (1H, d, J=7.5 Hz, anomeric H), 6.18 (1H, s, H-3), 6.81 (1H, s, H-7), 6.93 (1H, s, H-9), 7.18 (1H, s, H-10), 14.87 (1H, s, -OH), ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ: 183.68 (C-4), 168.70 (C-5), 161.82 (C-2), 161.02 (C-8), 157.58 (C-6), 152.38 (C-10a), 140.23 (C-9a), 107.66 (C-5a), 106.64 (C-3), 103. 57 (C-1′ & C-10), 101.13 (C-4a), 100.83 (C-7), 100.77 (C-8), 99.75 (C-1″), 76.82 (C-3′), 76.61 (C-5″), 76.30 (C-3″), 75.51 (C-5′), 73.47 (C-2′ & C-2″), 55.42 (-OCH_3), 20.09 (-CH_3).

Assay of radical scavenging activity

An 4ml of methanol solution of test extracts or co-

Table II. ^{13}C-NMR chemical shifts for anthraquinones in DMSO-d_6

Carbon	emodin	compound 7
C-1	161.51	150.24[a]
C-2	124.23	149.19[a]
C-3	148.35	122.92[b]
C-4	120.58	122.86[b]
C-5	108.84	108.51[c]
C-6	165.64	165.60
C-7	108.02	107.15[c]
C-8	164.54	164.35
C-9	189.83	190.09
C-10	181.48	179.89
C-11	132.91	131.28
C-12	113.46	113.92
C-13	109.06	108.98[c]
C-14	135.21	135.58
-CH_3	21.60	16.20
-OCH_3		

[a,b,c] may be reversed in each column

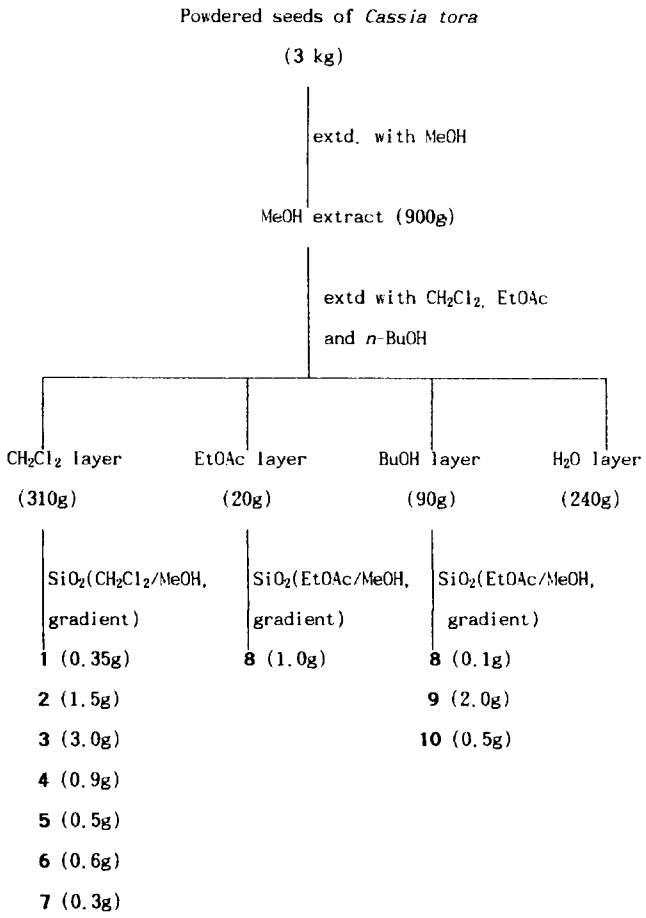

Chart 1. Fractionation and isolation of seeds of *Cassia tora*.

mpounds at various concentration (2.5-120 μg/ml) was added to a solution of DPPH (1.5×10^{-4} M) in MeOH (1 ml), and the reaction mixture was shaken vigorously. After storage at room temperature for 30 minutes in air, the remaining DPPH was determined by spectrophotometry at 517 nm. The radical scavenging activity (%) of each sample was expressed by the ratio of lowering of the absorption of DPPH, relative to the absorption of DPPH solution in the absence of test sample (control). The mean values were obtained from duplicate experiments.

RESULTS AND DISCUSSION

Structures of Isolated Compounds

Compound 7, obtained as yellow needles, mp 296-298℃, had the characteristic anthraquinone red color on the KOH reagent. The ^{1}H-NMR spectrum revealed a pair of doublet signals (J=2.0 Hz) at δ 7.08 and 6.52 ppm due to meta-coupling protons, a singlet signal at δ 7.47, a methyl signal at δ 2.34, and a characteristic hydroxyl signal at δ 12.02. The data suggested that the structure of **7** seems to be 2-hydroxy emodin(alaternin) isolated from *Rhamni cathartici* cortex (Rauwald and Just, 1981).

The identification of **7** as alaternin was finally established by comparison of ^{13}C-NMR spectral data with the known emodin (Danielsen *et al.*, 1992). The ^{13}C-NMR chemical shifts of **7** were similar to those of emodin except for the signals C-1, C-2 and C-3 due to the hydroxylation of C-2. From the above results, compound **7** was elucidated as 2-hydroxy emodin, alaternin.

Compound 8, yellowish powder, mp 257-259℃, had a methyl signal at δ 2.36 together with four aromatic protons at δ 6.12 (H-3), 6.72 (H-7 & H-9) and 7.15 (H-10) in the ^{1}H-NMR spectrum. In addition, a doublet at δ 4.90 (J=6.0 Hz) assigned to anomeric proton. Acid hydrolysis of compound 8 yielded an aglycone and D-glucose as the sugar. The aglycone was identified as nor-rubrofusarin by comparison of its spectroscopic data with the reported value (Kimura *et al.*, 1966). The ^{13}C-NMR data of compound 8 supported that glucopyranosyl moiety was attached to 6-position of nor-rubrofusarin. From these evidences, compound 8 was identified as nor-rubrofusarin-6-β-D-glucopyranoside(cassiaside) which was previously isolated from this plant (Wong *et al.*, 1989; Kimura *et al.*, 1966).

Compound 9, yellowish neddles, mp 186-188℃, had two anomeric proton signals at δ 5.05 (J=7.5 Hz) and δ 5.06 (J=7.5 Hz)ppm together with a methyl signal at δ 2.38, a methoxyl signal at δ 3.88 and four aromatic protons at δ 6.18 (H-3), 6.81 (H-7), 6.93 (H-9) and 7.18 (H-10) in the ^{1}H-NMR spectrum. Acid hydrolysis of compound 9 yielded an aglycone and D-glucose as the sugar. The aglycone was identified as rubrofusarin by comparison of its spectroscopic data with the reported value (Kaneda *et al.*, 1969). The ^{13}C-NMR data of compound 9 supported that disaccharide moiety was attached 6-position of rubrofusarin and interglycosidic linkage was shown to be glucose 1→6 glucose(gentiobiose) type by glycosidation shift for C-6 chemical shift of inner D-glucopyranose (Ushi *et al.*, 1973). On the basis of the above results, the structure of compound 9 was characterized rubrofusarin-6-β-D-gentiobioside which was also previously isolated from this plant (Wong *et al.*, 1989; Kaneda *et al.*, 1969).

Chrysophanol (compound 1), physcion (compound 2), β-sitosterol (compound 3), chryso-obtusin (compound 4), emodin (compound 5), aurantio-obtusin (compound 6) and chrysophanol triglucoside (compound 10) were identified by direct comparisons with anthentic samples and by spectral comparison (Wong *et al.*, 1988; Takido, 1960; Raghunathan *et al.*, 1974).

Radical Scavenging Activity

Active oxygen species such as superoxide radicals, hydrogen peroxide and hydroxyl radicals has been re-

Table III. Effects of various fractions from *Cassia tora* L.

Samples	50% reduc.[a] (μg)
MeOH	299.6
CH_2Cl_2	337.55
EtOAc	79.8
BuOH	146.25
H_2O	423.28
L-ascorbic acid	8.1
BHT	9.5

[a]Amount required for reduction of DPPH after 30 min

cognized as the principle agent responsible for the deterioration of polyunsaturated fatty acids, or lipid containing foods when exposed to air (Slater *et al.*, 1987). Lipid peroxidation is strongly associated with aging and carcinogenesis (Cutler, 1984).

To find the radical scavenging materials from the seeds of *Cassia tora*, the free radical scavenging activity was evaluated by the scavenging effect of the stable 1,1-diphenyl-2-picryhydrazyl radical spectrophotmetrically (Blois, 1958). A methanol solution of DPPH was found to be stable for over 60 min by colorimetry at 517 nm of an 80 μg/ml solution. The control intensity (absence of sample extracts) was taken as 100%, and the percentage intensity was calculated.

Table III shows the concentration for 50% inhibition of various fractions on the DPPH radical. Among the various fractions tested, ethyl acetate soluble fraction was the most potent scavenger of the DPPH radical and followed butanol and dichloromethane soluble fractions in order. The scavenging activities of the isolated compounds on DPPH radical are shown in Table IV. Among the ten isolated compounds, compounds 7, 8 & 9 exhibited higher scavenging activity on DPPH with IC_{50} value 17.59, 32.52 and 18.04 μg/4 ml.

Compound 7 isolated from the weak active CH_2Cl_2 fraction showed the most potent scavenging activity on DPPH. The concentration of this compound required for 50% inhibition was an approximate effect with that of L-ascorbic acid. The ability of alaternin to scavenge the DPPH radical was interesting because most anthraquinones are reported to make radicals rather than scavenge radicals. Anthraquinone compounds are the largest class of naturally occurring quinones and are widely distributed in lower and higher plants. Several research workers are only interested in the source of oxygen radical of these compounds (Kawasaki *et al.*, 1992). All the anthraquinones isolated, chrysophanol, physcion , chryso-obtusin, emodin, aurantio-obtusin and chrysophanol triglucoside except alaternin were not active on DPPH radical. These results show that the number of hydroxyl substituents are necessary for radical scavenging effect of anthraquinones. This was further clarified that emodin which lack of hydroxyl

Table IV. Effects of isolated compounds from *Cassia tora* L.

Samples	50% reduc.[a] (μg)
L-ascorbic acid	8.1
BHT	9.5
Alaternin	17.59
Nor-Rubrofusarin-glucoside	32.52
Rubrofusarin-6-β-gentiobioside	18.04
Aurantio-obtusin	>480
Chrysophanol	>480
Chryso-obtusin	>480
Physcion	>480
Emodin	>480
Chrysophanol-triglucoside	>480

[a]Amount required for reduction of DPPH after 30 min

group at C-2 has weak activity. The radical scavenging mechanism of naphthopyrones **8** and **9** on DPPH may be explained by the ring-opened products of these pyrones which may serve as nucleophiles to scavenge the radicals. However, definitive data are lacking whether this reaction would occur in methanol solution or not. Compound 10 isolated from the active BuOH fraction, showed no significant effect.

Radical scavenging effects of flavonoids, tannins and related polyphenols isolated from natural sources have been widely studied (Ushida *et al.*, 1987; Yoshida *et al.*, 1989), but the compounds isolated from *Cassia tora* have not been reported. It appears that the radical scavenging activity in the orignal methanol extract of *Cassia tora* was partially attributable to alaternin, cassiaside and ribrofusarin-6-β-D-gentiobioside. Our results suggest that the anthraquinone and naphthopyrone glycosides from *C. tora* may aid in the prevention and treatment of clinical disorder attributed to the reaction radicals. We have not further investigated the properties of these compounds. More detailed evaluation of the radical scavenging activity and structure-activity relationship of alaternin are in progress.

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