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Isolation and Identification of Antitumor Promoters from the Seeds of *Cassia tora*

Park, Yeung-Beom[1] and Seon-Bong Kim[2]*

[1]*Department of Food Processing and Bakery, Gangwon Provincial College, Gangnung 210-804, Korea*
[2]*Department of Food Science and Technology/Institute of Food Science, Pukyong National University, Busan 608-737, Korea*

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A methanol extract of *Cassia tora* seeds was successively partitioned with diethyl ether, chloroform, ethyl acetate, and water, and the antitumor-promoting activity of the solvent fractions was determined by inhibition of Epstein-Barr virus early antigen (EBV-EA) activation induced by teleocidin B-4 in Raji cells. The diethyl ether (68.7%) and chloroform (91.2%) fractions and the hydrolysate (94.3%) of the ethyl acetate fraction had strong inhibitory activities. The chloroform and ethyl acetate fractions were chromatographed on silica gel and further purified by HPLC. Three active compounds, obtusifolin-2-glucoside (75.0%), chryso-obtusin-6-glucoside (56.8%), and nor-rubrofusarin-6-glucoside (39.4%), were obtained from the ethyl acetate fraction, and two active compounds, questin (97.9%) and chryso-obtusin (53.8%), were isolated from the chloroform fraction.

Keywords: Epstein-Barr virus, antitumor promoter, *Cassia tora*, anthraquinone

Recent cancer survival rates have increased owing to advances in surgery, radiotherapy, and chemotherapy. However, cancer currently remains a leading cause of mortality world-wide [23]. Moreover, it has been the leading cause of death in Korea over the past 10 years, with incidence continuously increasing [10]. Unless cancer is detected and treated in its very early stages, the rate of full recovery is very low. Therefore, major research has been focused on chemoprevention as a means of cancer control, where induction can be totally prevented or the rate of development slowed or partially reversed.

The mechanism of chemical carcinogenesis has been explained by a multistage theory, which consists of initiation, promotion, and progression stages [3, 21]. The process of initiation involves irreversible reactions, is short-term, and is possibly unavoidable because of continuous exposure to carcinogenic chemicals, whereas promotion is a reversible long-term cumulative process involving repetitive exposure to a tumor promoter. Hence, inhibition of the promotion stage in multistage carcinogenesis has been regarded as a more efficient strategy to control and prevent cancer [3, 21]. Research on the control or prevention of cancer in the promotion stage has been executed using mainly plants, fruits, vegetables, and herbs that are known to have diverse pharmacological properties [17, 18]. Several active constituents such as catechins [26], naphthoquinones [6], flavanones [7], polyphenols [27], coumarins [5], flavonolignans [16], and β-carotene [20] have been reported as potent antitumor-promoting agents. *Cassia tora* is an annual herb belonging to leguminous weed species. Its soft and young leaves are used as edible vegetables, and roasted *Cassia* seeds are used to brew a tea.

Traditionally, the hot aqueous extract of *Cassia* seeds has been consumed in Korea as a healthy beverage, with such pharmacological actions as improvement of vision, protection of the liver, and diuretic, antiasthenic, and aperient effects [19]. In addition, *Cassia* seeds possess diverse physiological functions [8] including antifungal [11], antioxidant [25], antimutagenic [1], antigenotoxic [24], antiglycation [15], and antihepatotoxic effects [22], and the quinone-related compounds rhein, aloe-emodin, emodin, chrysophanol, rubrofusarin, *etc*. have been reported as the major pharmacological substances involved. In this study, in order to look for possible chemopreventive antitumor promoters, we carried out the isolation and identification of the active principles from *Cassia* seeds based on their ability to inhibit EBV-EA activation induced by teleocidin B-4, and also evaluated the structure–activity relationship of each compound isolated.

*Corresponding author
Phone: +82-51-629-5827; Fax: +82-51-629-5824;
E-mail: owlkim@pknu.ac.kr

Materials and Methods

Materials

Raji cells (CCL-86) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640 and fetal bovin serum (FBS) for the cultivation of cells were purchased from Gibco-BRL (Grand Island, NY, USA). The tumor promoter teleocidin B-4 and high-titer EBV-EA-positive sera from nasopharyngeal carcinoma (NPC) patients were kindly provided by Prof. Higashio of Kyoto University in Japan. Fluorescein isothiocyanate-immunoglobulin G (FITC-IgG) was purchased from DAKO (Glosrup, Denmark). Other chemicals, including *n*-butyric acid, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The chromatographic materials used were silica gel 60 and precoated Kieselgel $60F_{254}$ plates from Merck Co. (Darmstadt, Germany).

Instrumental Analysis

UV spectra were measured by a Shimadzu UV-160 spectrophotometer (Kyoto, Japan) with MeOH as the solvent. IR spectra were recorded on a Shimadzu IR-435 (Kyoto, Japan) in KBr pellets. ^{1}H-NMR spectra were scanned on a Varian Unity Plus 300 spectrophotometer (Palo Alto, CA, USA) in DMSO-d_6 using TMS as the internal reference.

Extraction and Fractionation with Methanol

Ground seeds of *Cassia tora* were extracted with 90% MeOH at ambient temperature for 12 h. The MeOH solution was then evaporated *in vacuo* at 40°C. The extract was resuspended in distilled H_2O and partitioned with diethyl ether, chloroform, ethyl acetate, and H_2O in sequence to give diethyl ether-, chloroform-, ethyl acetate-, and H_2O-soluble fractions, respectively (Fig. 1).

Separation and Identification of Active Fractions

The isolation and purification of antitumor promoters from solvent fractions is shown in Fig. 2. The ethyl acetate-soluble fraction was chromatographed on a silica gel column (2.7×53 cm), using chloroform-MeOH [from 10:0 to 0:10 (v/v)] as the eluent with an increasing proportion of MeOH. In order to check the degree of isolation, eluted fractions were monitored by TLC (chloroform-MeOH [8:2 (v/v)], sprayed with 15% H_2SO_4 reagent and heated) to generate two fractions with R_f =0.53 and R_f =0.64. These fractions were further purified by HPLC (Waters Associates HPLC system; detection, UV_{254}; mobile phase, 40% acetonitrile; flow rate, 1 ml/min) on semipreparative TSK gel ODS-120A (7.8×300 mm; Tosoh Co., Ltd., Tokyo, Japan) to produce compound A (T_R =5.70 min) from the fraction with an R_f value of 0.53, and compounds B (T_R =9.41 min) and C (T_R =10.55 min) from the fraction with an R_f value of 0.64. The chloroform-soluble fraction was subjected to column chromatography on a silica gel column using benzene-ethyl acetate as the eluting solvent [from 10:0 to 0:10 (v/v)] to give compounds D and E with an R_f value of 0.56 and 0.4, respectively, on TLC [benzene-ethyl acetate, 6:4 (v/v)].

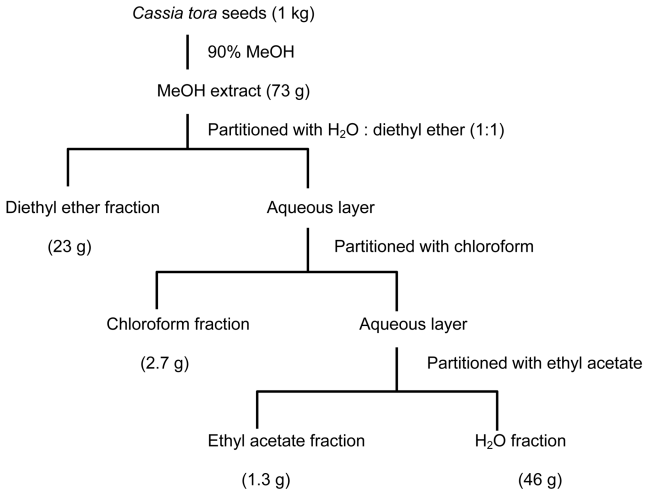


Fig. 1. Extraction and fractionation of active fractions from *Cassia tora* seeds.

Compound A (nor-rubrofusarin-6-glucoside). Yellow powder; UV (MeOH) λ_{max} nm: 400, 326, 277, 254, 225; IR (KBr) ν_{max} cm^{-1}: 3,400, 1,670, 1,570, 980–1,120; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.38 (3H, s, Me-2), 5.00 (1H, d, *J*=7.4 Hz, glucosyl H-1), 3.20–3.70 (6H, m, glucosyl-H), 6.16 (1H, s, H-3), 6.68 (1H, d, *J*=2.1 Hz, H-7), 6.72 (1H, d, *J*=2.1 Hz, H-9), 7.07 (1H, s, H-10), 14.9 (1H, s, OH-5), 10.3 (1H, s, OH-8).

Compound B (chryso-obtusin-2-glucoside). Yellow powder; UV (MeOH) λ_{max} nm : 355, 302, 278, 252, 214, 205; IR (KBr) ν_{max} cm^{-1}: 3,400, 1,670, 1,630, 1,580, 980–1,200; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.39 (3H, s, Me-3), 5.00 (1H, d, *J*=7.2 Hz, glucosyl H-1), 3.00–3.76 (6H, m, glucosyl-H), 3.870 (3H, s, OMe-7 or 8), 3.868 (3H, s, OMe-7 or 8), 3.88 (3H, s, OMe-1), 3.99 (3H, s, OMe-6), 7.50 (1H, s, H-5), 7.76 (1H, s, H-4).

Compound C (obtusifolin-2-glucoside). Yellow powder; UV (MeOH) λ_{max} nm: 402, 388, 372, 305, 275, 266, 210; IR (KBr) ν_{max} cm^{-1}: 3,400, 2,875, 1,630, 1,580, 980–1,200; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.33 (3H, s, Me-3), 5.29 (1H, d, *J*=7.4 Hz, glucosyl H-1),

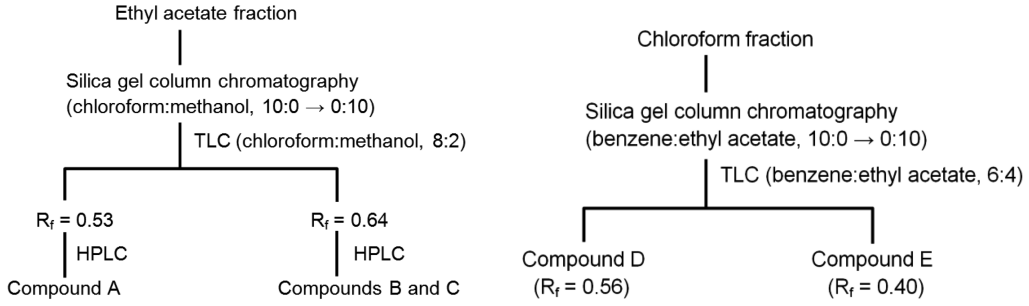


Fig. 2. Isolation and purification of antitumor promoters from solvent fractions of *Cassia tora* seeds.

3.20–3.76 (6H, m, glucosyl-H), 3.89 (3H, s, OMe-1), 7.35 (1H, d, *J*=8.0 Hz, H-5 or 7), 7.65 (1H, d, *J*=8.0 Hz, H-5 or 7), 7.76 (1H, dd, *J*=8.0, 8.0 Hz, H-6), 7.89 (1H, s, H-4).

Compound D (chryso-obtusin). Yellow powder; UV (MeOH) λ_{max} nm: 357, 306, 284; IR (KBr) ν_{max} cm^{-1}: 3,400, 2,920, 1,650, 1,570; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.27 (3H, s, Me-3), 3.81 (3H, s, OMe-1), 3.86 (3H, s, OMe-7), 3.88 (3H, s, OMe-8), 3.98 (3H, s, OMe-6), 7.50 (1H, s, H-5), 7.73 (1H, s, H-4).

Compound E (questin). Yellow powder; UV λ_{max} : 425, 284, 248, 223; IR (KBr) ν_{max} cm^{-1}: 3,400, 2,920, 1,650, 1,575; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 13.24 (chelated OH), 7.43 (1H, s, H-4), 7.21 (1H, d, *J*=1.9 Hz, H-5), 7.13 (1H, s, H-2), 6.85 (1H, d, *J*=1.9 Hz, H-7), 3.91 (3H, s, OMe-8), 2.27 (3H, s Me-3).

Inhibition of EBV-EA Induction

The inhibition of EBV-EA activation was assayed using Raji cells, non EBV-producing, EBV genome-carrying lymphoblastoid cells, which were cultivated in RPMI-1640 medium containing 10% FBS. The cells (1×10^6/ml) were incubated with 1 ml of medium containing *n*-butyric acid (3 mM, inducer), teleocidin B-4 (50 mM), and an amount of the test compound at 37°C in a 5%-CO_2 incubator. After 48 h, the reacted cell solution was centrifuged at 1,000 ×*g* for 10 min, and the medium was removed. Residual cells were redissolved in PBS buffer and this cell suspension was used for smears. Then, EA-induced cells were stained by a conventional indirect immunofluorescence technique with high-titer EBV-EA-positive sera from NPC patients followed by FITC-labeled IgG. In each assay, at least 500 cells were counted. EBV-EA inhibitory activity was estimated on the basis of the percentage of positive cells compared with that of control experiments with only *n*-butyric acid plus teleocidin B-4 without the test compound.

For the determination of cytotoxicity, cell viability was assayed by the trypan blue staining method.

RESULTS AND DISCUSSION

Antitumor-Promoting Activity of Solvent Fractions from Seeds of *Cassia tora*

A MeOH extract of *Cassia tora* seeds and solvent fractions successively partitioned from this extract were assayed for antitumor promoting activity using a short-term *in vitro* assay of EBV-EA activation induced by teleocidin B-4, and the results are presented in Table 1. In this assay, the MeOH extract had no inhibitory activity against EA induction. The inhibitory activity of each solvent fraction was significantly different and was in the order of chloroform (91.2%) > diethyl ether (68.7%) > ethyl acetate (24.7%) > water-soluble fraction (18.7%) at a concentration of 100 μl/ml. The comparative inhibitory activities of these fractions can be explained by their compositional differences and solubility. In particular, the nonpolar solvent fractions partitioned with chloroform and diethyl ether had better inhibitory activity compared with the polar ethyl acetate and H_2O fractions. Among the nonpolar solvent fractions, the chloroform fraction showed the strongest activity, with an inhibition rate and cell viability of 91.2% and 92.1%, respectively. Whereas the ethyl acetate fraction showed weak inhibitory activity (24.7%), it is interesting to note that the genin of the ethyl acetate fraction hydrolyzed by 2 N HCl at 140°C for 30 min exhibited a remarkably high inhibitory effect (94.3%) and cell viability (73.0%). Anthraquinones and their glycosides were expected to be present in the polar ethyl acetate fraction. From a healthy beverage point of view, which may be meaningful to consumers, most of the constituents in the ethyl acetate fraction are easily dissolved in water.

Table 1. Inhibitory effects of solvent fractions of *Cassia tora* seeds on teleocidin B-4-induced EBV-EA activation.

Fraction	Concentration (μg/ml)	Cell viability (%)	Inhibition (%)
Methanol	100	80.0	1.0
Diethyl ether	100	82.5	68.7
	20	74.2	26.7
Chloroform	100	92.1	91.2
	20	73.1	32.5
Ethyl acetate	100	73.3(73.0)[a]	24.7(94.3)
Water	100	84.4	18.7

[a]Ethyl acetate fraction was hydrolyzed with 2 N HCl at 140°C for 20 min.

Identification of Antitumor Promoters and Their Inhibitory Activities

Further detailed phytochemical investigations of the inhibition of EA induction by the ethyl acetate and chloroform fractions by silica gel column chromatography, TLC, and HPLC, as described in Materials and Methods, led to the isolation of nor-rubrofusarin-6-glucoside (compound A), chryso-obtusin-2-glucoside (compound B), and obtusifolin-2-glucoside (compound C) from the ethyl acetate fraction, and chryso-obtusin (compound D) and questin (compound E) from the chloroform fraction. Structural identification of these compounds was performed by UV, IR, and ^{1}H NMR, and the spectral assignments obtained were confirmed by comparing the data with those in prior literature [2, 4, 9, 15] (Table 2).

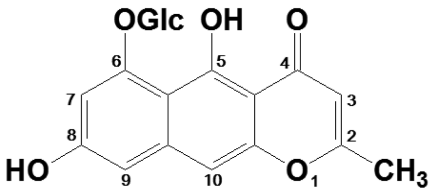
The chemical structures of naphthopyrone (A) and four anthraquinones (B–E) are presented in Fig. 3. The inhibitory activities of these five compounds against EA induction are shown in Fig. 4. Emodin has already been proved to have strong inhibitory activity against EA induction [12]. Among compounds A–E, questin (E), which is the 8-methyl ether of emodin, showed the strongest activity, with an inhibition rate and cell viability of 97.9% and 90.8%, respectively.

Konoshima *et al*. [12] reported that an anthraquinone in which the hydroxyl groups were evenly distributed on two phenyl rings exhibited greater inhibitory activity than an anthraquinone with unevenly distributed hydroxyl groups. Koyama *et al*. [13] also reported that 1,8-, 2,6-

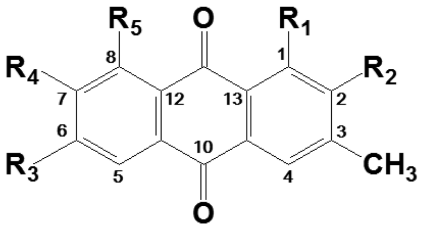
Table 2. ^{1}H NMR chemical shifts of compounds A–E.

H	Compound				
	A (A-1) [a]	B (B-1) [b]	C (C-1) [c]	D (D-1) [d]	E (E-1) [e]
H-2					7.13 (7.10)
H-3	6.16 (6.14)				
H-4		7.76 (7.75)	7.89 (7.86)	7.73 (7.68)	7.43 (7.41)
H-5		7.50 (7.48)	7.65 (7.64)	7.50 (7.46)	7.21 (7.16)
H-6			7.76 (7.74)		
H-7	6.68 (6.68)		7.35 (7.33)		6.85 (6.77)
H-9	6.72 (6.72)				
H-10	7.07 (7.04)				
CH_3-2	2.38 (2.36)				
3-CH_3		2.39 (2.39)	2.33 (2.44)	2.27 (2.26)	2.27(2.38)
1-OCH_3		3.88 (3.88)	3.89 (3.90)	3.81 (3.81)	
6-OCH_3		3.99 (3.98)		3.98 (3.97)	
7-OCH_3		3.87 (3.87)		3.86 (3.86)	
8-OCH_3		3.87 (3.87)		3.88 (3.87)	3.91 (3.88)
1,6-OH					13.24 (13.39)
5-OH	14.9 (14.9)				
8-OH	10.3 (10.3)				

[a,b,c,d,e] Data taken from references 15, 9, 2, 2, and 4, respectively.



Compound A (nor-rubrofusarin-6-glucoside)



Compound B–E

Compound	R_1	R_2	R_3	R_4	R_5
B (chryso-obtusin-2-glucoside)	OCH_3	OGlc	OCH_3	OCH_3	OCH_3
C (obtusifolin-2-glucoside)	OCH_3	OGlc	H	H	OH
D (chryso-obtusin)	OCH_3	OH	OCH_3	OCH_3	OCH_3
E (questin)	OH	H	OH	H	OCH_3

Fig. 3. Chemical structure of compounds isolated from *Cassia tora* seeds.

dihydroxyanthraquinone, where the hydroxyl groups were evenly distributed on two phenyl rings, showed stronger inhibitory activity than 1,2- and 1,4-dihydroxyanthraquinones. These reports suggest that the high inhibitory activity of questin may be related to the even distribution of hydroxyl groups on two phenyl rings. The inhibitory activity of compound B, which has an uneven distribution of methoxy groups on two phenyl rings at C-1, 6, 7, and 8, was less than that of compound C, which has one methoxy group. Therefore, from the viewpoint of structure–activity relationships, the number and distribution of methoxy groups may play an important role in the inhibition of EBV-EA induction.

Koyama *et al*. [14] reported a correlation between methylation of the phenolic hydroxyl groups in anthraquinone derivatives and inhibition of EA induction. They concluded that monomethylation of emodin led to a decrease in the inhibition rate, and emodin derivatives with more than two methoxy groups tended to increase the inhibitory effect.

In the present study, however, compound B with four methoxy groups was less active than compound C with

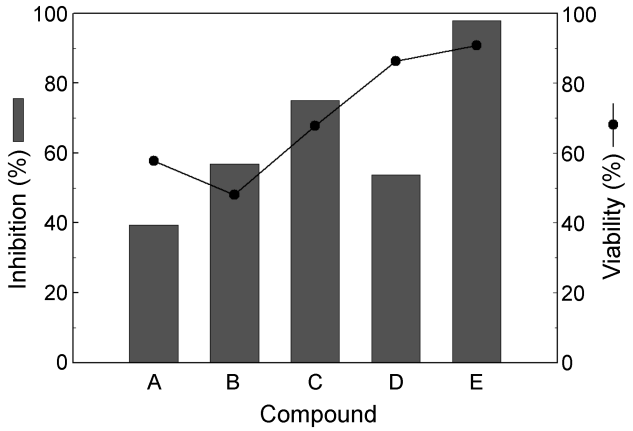


Fig. 4. Inhibitory activity of compounds A–E isolated from *Cassia tora* seeds at 100 μg/ml on teleocidin B-4-induced EBV-EA activation.

only one, implying that a number of methoxy moieties concentrated on one phenyl ring in the molecular structure seems to be related to the reduction in inhibitory activity against EBV-EA induction.

Meanwhile, the inhibitory rates of compounds B (56.8%) and D (53.8%) against EA induction were almost the same. With respect to cell viability, compound D (86.2%) showed a much higher rate than compound B (48.1%), its genin. It can be predicted that glycosides, in which a sugar is bound, will influence cell viability. However, as shown in Table 1, cell viability after treatment with the ethyl acetate fraction before and after hydrolysis was nearly the same, at around 73%. Additional research will be required in order to elucidate the relationship between cell viability and the presence of binding sugars.

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