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Anthraquinones with Immunostimulating Activity from *Cassia tora* L.

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Abstract

Many of plants had been reported having immunostimulating activity. This study reports the immunostimulating activity of *Cassia tora* L. (Leguminosae) seed, by means of solvent extraction method. Ethanol extract and solvent fractions, *n*-hexane, chloroform, ethylacetate, *n*-butanol and aqueous layer of *Cassia tora* L. seed were tested for immunostimulating activity *in vitro*. The ethylacetate-soluble fraction caused significant inhibition on the production of nitric oxide by murine macrophages (RAW 264.7), and mouse splenocytes were also stimulated at the concentration of 10 μg/mL. Three anthraquinones, chrysophanol (1), isochrysophanol (2) and aloe-emodin (3) with immunostimulating activity were isolated from the ethylacetate-soluble fraction of *Cassia tora* L. seed through activity-monitored fractionation and isolation method. These results permit *Cassia tora* L. to be useful as one the of natural immunostimulating crops.

Key words: *Cassia tora* L., Leguminosae, anthraquinone, nitric oxide, macrophage, splenocyte, phytochemicals, *in vitro*

INTRODUCTION

Bioactive compounds in plant foods are considered to be critical for human health. Plant is a proven source of numerous phytochemical agents and secondary metabolites, and it is reasonable to believe that there are additional agents in existence that remain undiscovered. (1).

Macrophages are important cells that play important roles in immune-system defense including phagocytosis of pathogens, production of many cytokines, proteolytic processing, and presentation of foreign antigens. In addition to cytokines, nitric oxide (NO) has been accepted as the mediator that has similar functions to these cytokines. The NO may exert the antimicrobial effect, inhibiting the replication of several viruses or parasites (2,3). To develop new material that maintains immunostimulating activity, many attempts have been achieved (4,5).

Cassia tora L. (Leguminoceae) which is grown widely in South Korea, have been identified with various types of bioactive phytochemicals, anthraquinones, anthraquinone glycosides, naphthoquinones, steroids and flavones with antioxidant, anti-mutagenic, anti-inflammatory, anti-fungal, antibacterial, antipyretic and hypoglycemic effects (6-12).

For the finding of biological importance of *Cassia tora* L., as one of the nutraceutical crops, three anthraquinones, having inhibitory activities on NO in murine peritoneal macrophages were isolated from ethylacetate (EtOAc) soluble fraction of *Cassia tora* L. seed. Compounds were found to exhibit inhibition of NO synthesis by RAW 264.7 cells *in vitro*.

MATERIALS AND METHODS

Plant material

The dried seed of *Cassia tora* L. (Leguminosae) was purchased at Kyungdong traditional medicine market, Seoul, Korea in April, 2004.

Chemicals

Complete RPMI 1640 medium (Gibco, Invitrogen Co., Carlsbad, California, USA) and FBS (Bio Whittaker, Cambrex Co., Walkersville, Maryland, USA) were used. All other chemicals were purchased from commercial sources and were of the highest purity available.

Instrumental analyses

Melting points (mp) were determined using a Mitamura-Riken melting point apparatus and are uncorrected. Electron impact mass spectrometry (EI-MS) spectra were obtained on a Hewelett Packard Model 5985B Gas chromatography (GC)/MS system. The Ultraviolet (UV)/Visible and Infrared (IR) spectra were recorded on a

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Hitachi 3100 UV/Vis and JASCO Fourier transform (FT)-IR-5300 spectrophotometer, respectively. A Bruker AMX500 spectrometer was used to record nuclear magnetic resonance (NMR) spectra (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS), and DMSO-d_6 as an internal standard and NMR solvents, respectively.

General experiment

Thin-layer chromatographic (TLC) analysis was performed on silica gel (Kieselgel 60 F_{254} plates (0.25 mm layer thickness; Merck, Darmstadt, Germany), with compounds visualized by spraying with 10% KOH-methanol (MeOH) after developing samples. Silica gel (Merck 60 A, 230~400 mesh ASTM) and Sephadex LH-20 (25~100 μm; Pharmacia Fine Chemicals, Piscataway, NJ, USA) were used for open column and vacuum column chromatographic separation.

Extraction and isolation of bioactive compounds

The dried seed of *Cassia tora* L. (1.2 kg) was extracted with ethyl alcohol (EtOH) for three times for three hours at hot water bath. The combined ethanolic extracts were partitioned between *n*-hexane and water, with the more polar layer then partitioned with chloroform ($CHCl_3$), EtOAc and *n*-butanol (*n*-BuOH). The fractions were bio-assayed before additional chromatographic fraction, then, fractions with the desired activity were applied for the isolation of bioactive components. The elutes on condensation resulted as a solid material and further purified by re-crystallization with highly-purified MeOH to give the pure compounds **1**, **2** and **3**.

Cell culture

Murine macrophage-like cell line (RAW 264.7 cells) and splenocytes were grown in RPMI 1640 containing 2 mM glutamine, 10% heat-inactivated fetal calf serum, penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in 5% CO_2 (13).

NO production

The amount of NO in the cultured medium of macrophages was determined as nitrite, a stable end product of NO. Cultured RAW 264.7 cells were treated with 1% trypsin and washed three times with serum free RPMI 1640 medium (300×g, 5 min). Cells were added to 24-well multiplate with the concentration of 2.0×10^5 cells/mL. After culture for 48 hour at 37°C and 5% CO_2, cell were centrifuged for 30 min at 400×g. A 100 μL of supernatant was transferred to ELISA titer plate. The 100 μL of Griess reagent (1:1 mixture (v/v) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4) was added in each well, and mixed well. The mixture was left for 10 min at room temperature and the absorbance was measured at 540 nm by a microplate reader. The nitrite concentration was quantified from the standard curve with $NaNO_3$.

Preparation of splenocytes

Erythrocytes in splenocytes were disrupted with ACK lysing buffer (8.29 g/NH_4Cl, 1.0 g/$KHCO_2$, 37.2 mg/L EDTA·2Na). Splenocytes were maintained in RPMI 1640 medium supplemented with 5.0 mg/mL gentamycin sulfate containing 10% heat inactivated fetal calf serum.

Alkaline phosphatase activity

The cell lysates were measured for alkaline phosphatase (ALP) activity to estimate the effect of *Cassia tora* L. on splenocyte. Colorimetric assays have been used for the assessment of ALP activity. The splenocytes were treated with various concentration of *Cassia tora* L. seed in 24-well plate and cultured for 48 hr at a density of 1.0×10^6 cells/mL in a 5% CO_2 incubator at 37°C. The cell suspensions were collected and freeze-thawed. A 100 μL *p*-nitrophenylphosphate 2Na dissolved in 10% diethanolamine-HCl was added to 25 μL of cell lysate. The reaction mixture was incubated at 37°C for 60 min and the optical density at 405 nm was measured. The stimulation index (SI) of the assay was defined as the ratio of the absorbance signal in control and stimulated cells and calculated as follows:

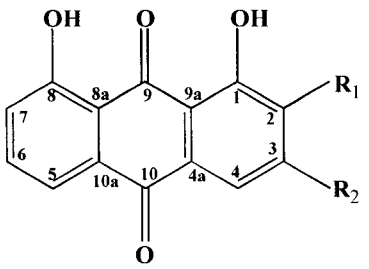
$$SI = (S-C)/C$$

where *S* and C represent the absorbance values for the samples and control cells, respectively.

RESULTS AND DISCUSSION

Isolation and structure elucidation of compounds

The EtOAc-soluble fraction of seed of *Cassia tora* L. has been chromatographed over a silica gel column using a $CHCl_3$-MeOH gradient to give seven sub-fractions monitoring TLC patterns on UV lamp and 10% KOH-MeOH spray. The EtOAc-soluble fraction caused significant inhibition on macrophages cell line (murine RAW 264.7), and mouse splenocytes were also stimulated at the concentration of 10 μg/mL, of these, subfractions 3 and 5, which possessed immunostimulating activity with IC_{50} values of 58.6 and 75.8 μg/mL, were further chromatographed on a silica gel and Sephadex LH-20 column by elution with $CHCl_3$-MeOH (93:7) and MeOH in order to give pure compounds **1**, **2** and **3**. Complete identification of isolated compounds made use of varieties of physical and chemical methods, which includes EI-MS spectrometry, UV/Vis and IR Spectrophotometer, and ^{1}H-NMR and ^{13}C-NMR spectroscopy. The structures of compounds (Fig. 1) were identified by com-



1 : R_1=H; R_2=CH_3
2 : R_1=CH_3, R_2=CH_2OH
3 : R_1=H, R_2=CH_2OH

Fig. 1. Chemical structure of isolated compounds **1**, **2** and **3** from *Cassia tora* L. seed.

paring spectra with published data (14,15).

The isolated compounds were determined as chrysophanol (**1**), isochrysophanol (**2**) and aloe-emodin (**3**), and detailed data is described as follows;

***Chrysophanol* (*1*)**; *1,8-Dihydroxy-3-methylanthraquinone* ($C_{15}H_{10}O_4$) : Yellow plates from MeOH; mp 254~252 °C; UV λ_{max} (MeOH) (log ε) : 225 (4.70), 258 (4.54), 279 (4.73), 288 (4.19), 434 (3.94) nm; IR (KBr) ν_{max} 3400 (OH), 2890 (CH), 1670, 1620 (C=O), 1612, 1510 (aromatic C=C) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity, %): 254 $[M]^+$ (27.0), 236 $[M-H_2O]^+$ (12.5), 221 $[M-H_2O-CH_3]$ (12.6), 218 $[M-2H_2O]^+$ (23.4); ^{1}H-NMR and ^{13}C-NMR data were consistent with those in the literature (14,15), described as Table 1.

***Isochrysophanol* (*2*)**; *1,8-Dihydroxy-2-methylanthraquinone* ($C_{16}H_{12}O_5$) : Yellow needles from MeOH; mp 179~180 °C; UV λ_{max} (MeOH) (log ε) : 223 (4.79), 257 (4.51), 276 (4.70), 289 (4.19), 435 (3.98) nm; IR (KBr) ν_{max} 3408 (OH), 2890 (CH), 1670, 1620 (C=O), 1612, 1512 (aromatic C=C) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity, %): 285 $[M+1]^+$ (100.0), 284 $[M]^+$ (46.7), 254 $[M+1-CH_2OH]^+$ (8.2), 218 $[M-2H_2O]^+$ (13.4), 203 $[M-2H_2O-CH_3]^+$ (52.9); ^{1}H-NMR and ^{13}C-NMR data were consistent with those in the literature (14,15), described as Table 1.

***Aloe-emodin* (*3*)**; *1,8-Dihydroxy-3-hydroxymethylanthraquinone* ($C_{15}H_{10}O_5$) : Orange needles from MeOH; mp 224~226°C; UV λ_{max} (MeOH) (log ε) : 225 (4.70), 258 (4.54), 276 (4.73), 288 (4.19), 430 (3.94) nm; IR (KBr) ν_{max} 3410 (OH), 2890 (CH), 1670, 1620 (C=O), 1612, 1512 (aromatic C=C) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity, %): 270 $[M]^+$ (11.0), 252 $[M-H_2O]^+$ (32.7), 242 $[M-CO]^+$ (17.5), 240 $[M+1-CH_2OH]^+$ (56.1), 224 $[M-CO-H_2O]^+$ (28.4); ^{1}H-NMR and ^{13}C-NMR data were consistent with those in the literature (14,15) described as Table 1.

Immunostimulating activity of *Cassia tora* L.

Cassia tora L. seed had been investigated to confirm the effect on the NO production and ALP activities by murine peritoneal macrophages and splenocytes. Murine peritoneal macrophages and splenocytes were chosen for their potential ability to enhance the immune responses. Macrophages generate the NO, one of reactive oxygen species through partial reduction of oxygen.

The macrophages, exposed to EtOAc-soluble fraction of *Cassia tora* L. seeds, produced increasing amounts

Table 1. ^{1}H-NMR and ^{13}C-NMR data for compounds **1**, **2** and **3** from *Cassia tora* L. seed

Position	$\delta^1H^{1)}$			$^{13}C^{2)}$		
	1	2	3	1	2	3
1				162.6	161.9	161.7
2	7.27		7.21	120.9	156.2	120.1
3				151.9	151.3	151.4
4	7.68	7.69	7.69	117.1	117.3	117.3
5	7.80	7.82	7.81	118.6	118.4	118.6
6	8.41	8.43	8.42	138.2	138.5	138.3
7	6.77	6.81	6.78	115.1	115.0	115.4
8				162.2	161.5	162.0
9				192.5	192.3	193.2
10						
4a				131.4	131.5	131.2
8a				116.5	116.4	116.5
9a				115.0	115.2	115.1
10a				133.1	133.0	133.1
CH_3	1.73	1.70		17.8	17.5	
CH_2OH		4.59	4.39		62.6	62.2
OH-1	12.57	12.54	12.59			
OH-8	12.45	12.41	12.40			

[1)]TMS was used as the internal standard; chemical shifts are shown in the δ scale; measured at 500 MHz in DMSO-d_6.
[2)]Measured at 125 MHz in DMSO-d_6.

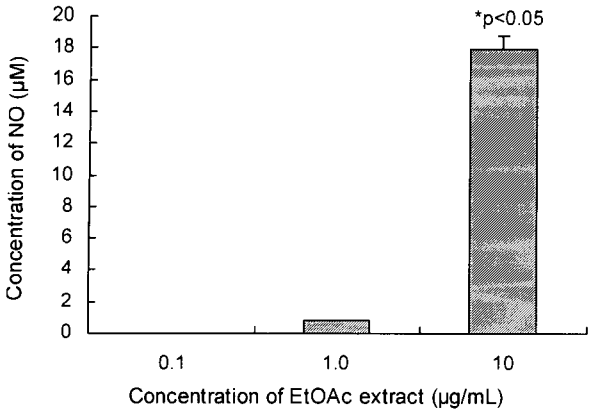


Fig. 2. NO concentration of RAW 264.7 macrophage to the variable concentration of EtOAc extract of *Cassia tora* L. seed.

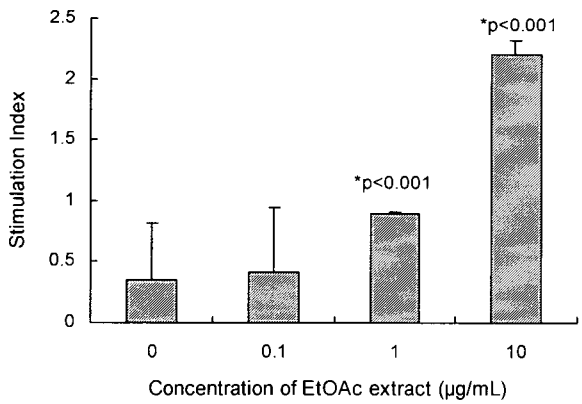


Fig. 3. Alkaline phosphatase activities of splenocytes to the variable concentration of EtOAc extract of *Cassia tora* L. seed.

of nitrite with the concentrate-dependant manner. At the concentration of 10 μg/mL, the production of nitrite was potentially stimulated by EtOAc-soluble fraction (Fig. 2). ALP on murine splenocytes also showed correlated activities with the same concentration of EtOAc-soluble fraction (Fig. 3). On the other hand, other solvent fractions, $CHCl_3$ and *n*-BuOH extracts showed no inhibitory effect on the synthesis of NO and ALP activities in same concentrations.

To determine the bioactivity of compounds from EtOAc-soluble fraction, we isolated three anthraquinones (**1**, **2**, and **3**) through silica gel and Sephadex LH-20 column chromatographic method. At the concentration of 10 μg/mL, the production of nitrite of compounds **1**, **2** and **3** were 27.8, 20.9 and 29.4 μM, respectively. As a positive control, L-NMMA (NO synthesis inhibitory agent) showed significant inhibition with the production of nitrite at 12.5 μM (Table 2).

Anthraquinone may either be formed via the acetate pathway or by a sequence involving shikimic acid and mevalonic acid pathways. Anthraquinone from plants were also reported to hold immunoenhancing effects such as the activation of cytokine production. Emodin isolated from *Polygonum hypoleucum* Ohwi decreased cytokine production and IL-2 mRNA expression (16,17). Screening of plant extracts and its solvent fractions for identification of bioactive components that could effectively induce immunity on some promising preventive and/or controlled candidates for degenerative diseases.

Table 2. NO concentration of Raw 264.7 macrophage of compounds **1**, **2** and **3**

Compounds[1)]	Concentration of NO (μM)
1	27.8±0.47
2	20.9±0.44*[2)]
3	29.4±0.65
L-NMMA[3)]	12.5±0.31*

[1)]Each samples were tested at concentration of 10 μg/mL.
[2)]Significant level of at $p<0.05$.
[3)]Positive control.

From these results, we investigated that compounds **1**, **2** and **3** could stimulate a nonspecific immune response of macrophages, and the supplementation of *Cassia tora* L. seed might be to offer health benefit in immune system. *In vivo* evaluation of compounds on inhibition on macrophages cell line and mouse splenocytes remains to be carried out.

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