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Anthraquinones of edible wild vegetable *Cassia tora* stimulate proliferation of human CD4$^+$ T lymphocytes and secretion of interferon-gamma or interleukin 10

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Abstract

Cassia tora L. is an edible wild plant. This study evaluated the immunostimulatory activities of four anthraquinones of *C. tora* (aloe-emodin, emodin, chrysophanol, and rhein) on human peripheral blood mononuclear cells (PBMC). Studies were conducted on lymphocyte proliferation by BrdU immunoassay, secretion of interferon-gamma (IFN-γ) and interleukin 10 (IL-10) by an ELISA assay and elucidation of responding immune cells by flow cytometry. The results showed that at non-cytotoxic concentrations, the tested anthraquinones were effective in stimulating the proliferation of resting human PBMC and/or secretion of IFN-γ. However, at the concentration of 10 μg/ml (35 μM), rhein significantly stimulated proliferation of resting human PBMC (stimulation index (SI) = 1.53), but inhibited IFN-γ secretion (74.5% of control). The augmentation of lymphocyte proliferation was correlated to the increase in number of CD4$^+$ T cells, while the elevated secretion of IFN-γ and IL-10 might have been due to the activated CD4$^+$ T cells.

Keywords: Anthraquinones; Lymphoproliferation; Interferon-gamma (IFN-γ); Interleukin 10 (IL-10); CD4$^+$ T lymphocytes

1. Introduction

Cassia tora L. is an edible wild plant (Iwasa, 1980; Jeng, 1992; Jeng & Chang, 1981; Liu, Chou, & Ou, 1978). The young leaves and immature pods of *C. tora* are consumed as a vegetable and its toasted mature seeds are popularly used as a healthy beverage in Asia (Hsu & Chiu, 1986; Jeng, 1992; Jeng & Chang, 1981; Jou, 1987; Lin, 2003; Liu et al., 1978; Wu, Hsieh, Song, & Yen, 2001; Yen & Chuang, 2000; Yen & Chung, 1999). In addition, seeds of *C. tora* possess physiological function as an antioxidant, diuretic, diarrheal, antiseptic, and antimutagen (Choi, Lee, Park, Ha, & Kang, 1997; Jou, 1987; Kitanaka & Takido, 1986; Wu et al., 2001; Yen, Chen, & Duh, 1998; Yen & Chuang, 2000; Yen & Chung, 1999). Anthraquinones (aloe-emodin, emodin, chrysophanol, rhein) are the major types of phenolic compounds that are present in this wild

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; Con A, concanavalin A; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; FITC, isothiocyanate; IFN-γ, interferon-gamma; IL-10, interleukin 10; mAbs, monoclonal antibodies; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PHA, phytohemagglutinin; SI, stimulation index.

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vegetable *C. tora* and many traditional Chinese medicinal plants associated with anticancer activities including *Polygonum cuspidatum* Sieb. et Zucc., *Polygonum multiflorum* Thunb. and *Rheum officinale* Baill (Cai, Luo, Sun, & Corke, 2004). In the literature, many studies have indicated that the above compounds have antitumor or antimetastasis effects (Kuo, Hsu, Ng, & Lin, 2004; Lee, 2001; Zhang et al., 1998). In addition, emodin has been reported to possess other biological activities such as antigenotoxicity (Wu et al., 2001), anti-mitosis of activated T cells (Huang et al., 1992), anti-proliferation of activated mesangial cells (Kuo et al., 2001a) and stimulation of nucleotide excision repair (Chang, Sheu, Huang, Tsai, & Kuo, 1999). In some studies, rhein has also been demonstrated to possess antigenotoxicity (Wu et al., 2001) and antiinflammation (Martel-Pelletier, Mineau, Jolicoeur, Cloutier, & Pelletier, 1998). With regard to chrysophanol, a number of studies have indicated that it has antigenotoxic (Wu et al., 2001), antimutagenic (Choi et al., 1997), antiallergic (Kim, Park, Bae, & Ham, 2000) and antiviral effects (Semple, Pyke, Reynolds, & Flower, 2001).

In a previous study, the crude extracts of *C. tora* seeds were shown to possess significant immunostimulating effects on resting mononuclear cells. In this study, the aim was to investigate the immunostimulating effects of the above four anthraquinones on resting mononuclear cells by lymphoproliferation test and to define the responding cells by flow cytometry and secretion of multipotent immune activator IFN-γ and inhibitor IL-10 by ELISA method. The structure-activity relationship was also elucidated.

2. Materials and methods

2.1. Chemicals

Heparin, Ficoll–Hypaque, dimethylsulfoxide (DMSO), phytohemagglutinin (PHA), concanavalin A (Con A), chrysophanol, rhein, aloe-emodin and emodin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The purity of anthraquinones was more than 98% as determined by HPLC analysis. 5-Bromo-2′-deoxyuridine (BrdU) labeling and detection kits were obtained from Roche Diagnostics GmbH (Mannheim, Germany). The human IFN-γ and IL-10 immunoassay kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA).

2.2. Lymphoproliferation test

Peripheral blood from eight healthy volunteers aged 20–35 years old was collected in a sterile syringe containing sufficient heparin to provide a final concentration of 100 units/ml. Mononuclear cells were obtained by centrifuging (25 °C, 400 g, 30 min) of the mixture of blood and normal saline (1/1, v/v) on Ficoll–Hypague (2.4:1) gradients as described by the manufacture's protocol (Sigma-Aldrich). After centrifugation, 0.05 ml (5.0×10^5) unfractionated PBMC was placed in a 5 ml test tube containing 0.15 ml fetal calf serum and 0.75 ml of RPMI 1640, with 0.05 ml of test samples, mitogen (positive control: PHA, ConA) (Severinson & Larsson, 1986), medium (negative control) or additional medium with DMSO (solvent control). After gentle mixing, 200 μl were added into the wells of a 96-well microculture plate in triplicate. The culture plate was then allowed to incubate for 3 days at 37 °C in a 5% CO_2 incubator. The BrdU immunoassay was completed as previously described (Chiang, Ng, Chiang, Chang, & Lin, 2003). The optical densities were determined with an ELISA reader at a test wavelength of 450 nm. The stimulation index (SI) was determined by the ratio of optical density of test substance to the optical density of negative control.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The ELISA procedure was performed according to the standard protocol of the ELISA kit. The cultivation and treatment of human PBMC was completed as previously described in the lymphoproliferation test. After 3 days, particulates were removed from the supernatants by centrifugation, and the samples were stored at −70 °C until used. The sample diluent (100 μl) was added to each well and then 100 μl/well of cytokine standard or supernatant sample was applied. The plate was then covered with an adhesive strip and incubated for 2 h at 37 °C. Each well was aspirated and washed, and the process was repeated four times. The cytokine conjugate (200 μl/well) was added, covered with a new adhesive strip, and incubated for 2 h at 37 °C. The washing process was repeated four times. The substrate solution (200 μl/well) was added and incubated for 30 min at 37 °C. Finally, the stop solution (50 μl/well) was added and the optical density of each well determined within 30 min, using an ELISA reader at a test wavelength of 450 nm and a reference wavelength of 540 nm.

2.4. Flow cytometry analysis

All fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) were purchased from Becton Dickinson (San Jose, CA, USA). Optimal concentrations of mAbs were determined for each mAb by titration. The isolated PBMC were cultured in triplicate with various concentrations of test drugs, PHA (5 μg/ml), DMSO (0.1%) or medium for 3 days at 37 °C in a 5% CO_2 incubator. Then the numbers of NK cells ($CD3^-$, $CD16^+$, $CD56^+$), activated PBMC ($CD25^+$), T cell subsets ($CD3^+$, $CD4^+$, $CD8^+$), total T cells ($CD3^+$), total B cells ($CD3^-$, $HLA\text{-}DR^+$) and active T cells ($CD3^+$, $HLA\text{-}DR^+$) at 0 or 3 days were determined by standard FACScan procedures with mAbs according to the manufacture's protocol. In brief, at the indicated time period, the cultured cells were stained with 20 μl of mAb for 30 min at 25 °C. The stained cells were centrifuged

(25 °C, 300 g, 5 min.) and the supernatant was aspirated leaving 50 μl of residual fluid in the tube. The cell pellet was resuspended and then 2 ml of PBS was added to each tube. Each tube was vortexed thoroughly at low speed for 3 sec and then centrifuged (25 °C, 200 g, 5 min). After aspiration of the supernatant until there was 50 μl of residual fluid left, the cell pellet was resuspended and then 0.5 ml of 1% paraformaldehyde was added to each tube. Finally, the stained PBMC were analyzed by a flow cytometric analyzer (FACSCalibur, Becton Dickinson, Cookeysville, MD, USA) with Cell Quest software (Becton Dickinson).

2.5. Statistical analysis

Results were expressed as mean ± standard error. The one-way ANOVA and multiple comparison of Dunnett's *t*-test (>2 groups) or 2-tails of Student's *t*-test (2 groups) were used to evaluate the difference between the control and test samples by the SPSS Base 8.0 software for Windows. A p value of less than 0.05 was considered to be a significant difference in all experiments.

3. Results

3.1. Lymphocyte proliferation and secretion of multipotent immune activator IFN-γ

When exploring the immunostimulating phytochemicals, results showed that at concentrations lower than 10 μg/ml, four anthraquinones significantly stimulated the proliferation of resting PBMC, whereas 1 μg/ml of aloe-emodin and emodin augmented the secretion of IFN-γ (Table 1). IFN-γ secretion has been well-known to be associated with lymphocyte activation after stimulation by antigens or mitogens, and PHA and Con A showed this typical response. However, there were three other types of immunostimulation after treatment with anthraquinones. Type 1 of rhein stimulated resting lymphocyte proliferation and inhibited IFN-γ secretion. Aloe-emodin and emodin expressed a type 2 immunostimulation, which stimulated the proliferation of resting PBMC at high doses (10 μg/ml) and augmented the secretion of IFN-γ at low doses (1 μg/ml). However, type 3 immunostimulation of chrysophanol enhanced the proliferation of resting PBMC but did not stimulate IFN-γ secretion (Tables 1 and 2).

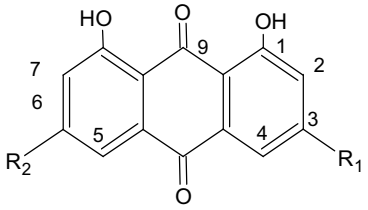
Table 1
The immunomodulatory activity of four anthraquinones

Drug	Dose (μg/ml)	Stimulation index[a]	γ-Interferon (pg/ml)[a]
DMSO	0.10%	1.00 ± 0.02	11.8 ± 1.14
Con A[b]	10	5.98 ± 0.58*	523 ± 47*
PHA[b]	5	5.56 ± 0.38*	541 ± 36*
Aloe-emodin	1	1.24 ± 0.20	15.5 ± 1.37*
	10	1.71 ± 0.22*	10.6 ± 1.12
Emodin	1	1.26 ± 0.23	14.7 ± 1.32*
	10	1.29 ± 0.15*	11.5 ± 1.21
Chrysophanol	1	1.47 ± 0.22*	13.3 ± 1.35
	10	1.35 ± 0.21*	12.5 ± 1.37
Rhein	1	1.35 ± 0.25*	12.6 ± 1.41
	10	1.53 ± 0.26*	8.8 ± 0.74*

[a] The drugs were evaluated on their ability to direct stimulation of PBMC without mitogen in triplicate on eight volunteers. One-way ANOVA and multiple comparison of Dunnett's *t*-test were used to evaluate the difference between test drug and DMSO control (*$p < 0.05$).

[b] The positive control: concavalin A (Con A), phytohemagglutinin (PHA).

Table 2
Structures of anthraquinones and their immunostimulatory activity



Compound	R1	R2	Activity[a]
Rhein	COOH	H	1,2
Chrysophanol	CH_3	H	2
Emodin	CH_3	OH	2,3
Aloe-emodin	CH_2OH	H	2,3

[a] Immunostimulatory pattern: type 1 (enhanced lymphocyte proliferation and inhibited secretion of IFN-γ), type 2 (enhanced lymphocyte proliferation) and type 3 (enhanced secretion of IFN-γ).

3.2. Detection of responding cells

To determine which types of lymphocytes to direct the patterns of immunostimulation, we used flow cytometric analysis. The results from the control groups showed that medium control decreased the fraction of total B cells while solvent control increased the fraction of active T cells (Table 3). The immunostimulatory effects by PHA exhibited significant elevation of $CD8^+$ T and activated PBMC, whereas the $CD4^+$ T and total B cells were slightly

Table 3
The cell fractions of controls

Cells[a]	Day 0	Day 3	
		Medium	DMSO (0.1%)
Total T	0.597 ± 0.066	0.595 ± 0.053	0.609 ± 0.065
Total B	0.229 ± 0.021	0.175 ± 0.012*	0.216 ± 0.042
Active T	0.128 ± 0.029	0.139 ± 0.015	0.271 ± 0.029*
$CD4^+$ T	0.341 ± 0.036	0.391 ± 0.026	0.307 ± 0.066
$CD8^+$ T	0.404 ± 0.042	0.379 ± 0.021	0.421 ± 0.041
NK cell	0.296 ± 0.067	0.318 ± 0.055	0.336 ± 0.063
Activated PBMC	0.060 ± 0.008	0.092 ± 0.010	0.075 ± 0.013

[a] Total T ($CD3^+$), Total B ($CD3^-$, HLA-DR^+), Active T ($CD3^+$, HLA-DR^+), $CD4^+$ T ($CD3^+$, $CD4^+$), $CD8^+$ T ($CD3^+$, $CD8^+$), NK cell ($CD3^-$, $CD16^+$, $CD56^+$), Activated PBMC ($CD25^+$).

* The medium or DMSO control were evaluated on their ability to stimulate resting PBMC in triplicate on eight healthy volunteers and determine the difference between day 0 and day 3 (*$p < 0.05$).

decreased. The type 1 immunostimulation by rhein showed that it significantly increased the $CD4^+$ T cells and decreased total B, active T and $CD8^+$ T cells. Although aloe-emodin exhibited type 2 immunostimulation, it induced similar changes to the fractions of immune cells as rhein had induced. The type 3 immunostimulation by aloe-emodin showed that it significantly increased $CD4^+$ T cells, whereas it decreased total B and active T cells (Table 4). Induction of multipotent cytokines IFN-γ and IL-10 production by aloe-emodin indicated that it might be due to increase Th1 and Th2 of $CD4^+$ cells (Tables 4 and 5).

Table 4
The cell fractions at day 3 after treatment with controls or anthraquinones

Cells[a]	DMSO (0.10%)	PHA[b] (5 μg/ml)	Rhein[c] (10 μg/ml)	Aloe-emodin[d] (10 μg/ml)	Aloe-emodin[e] (1 μg/ml)
Total T	0.609 ±0.065	0.632 ±0.056	0.600 ±0.061	0.580 ±0.062	0.578 ±0.074
Total B	0.216 ±0.042	0.173 ±0.032	0.116* ±0.01	0.119* ±0.019	0.154* ±0.019
Active T	0.271 ±0.029	0.262 ±0.016	0.080* ±0.011	0.085* ±0.012	0.096* ±0.013
$CD4^+$ T	0.307 ±0.066	0.273 ±0.029	0.412* ±0.029	0.400* ±0.031	0.393* ±0.039
$CD8^+$ T	0.421 ±0.041	0.513* ±0.053	0.349* ±0.022	0.339* ±0.026	0.364 ±0.029
NK cell	0.336 ±0.063	0.323 ±0.045	0.313 ±0.041	0.303 ±0.055	0.314 ±0.052
Activated PBMC	0.075 ±0.013	0.344* ±0.003	0.096 ±0.011	0.070 ±0.011	0.087 ±0.011

The study was evaluated on anthraquinones direct stimulation of resting PBMC without mitogen in triplicate of eight healthy volunteers. The type 1 response increased lymphocyte proliferation and decreased IFN-γ secretion; type 2 response enhanced lymphocyte proliferation; and type 3 response elevated IFN-γ secretion.

[a] Total T ($CD3+$), Total B ($CD3^-$, $HLA\text{-}DR^+$), Active T ($CD3^+$, $HLA\text{-}DR^+$), $CD4^+$ T ($CD3^+$, $CD4^+$), $CD8^+$ T ($CD3^+$, $CD8^+$), NK cell ($CD3^-$, $CD16^+$, $CD56^+$), Activated PBMC ($CD25^+$).

[b] The positive controls were compared with solvent (DMSO) control ($*p < 0.05$).

[c] The type 1 immunostimulation of rhein was compared with DMSO ($*p < 0.05$).

[d] The type 2 immunostimulation of aloe-emodin was compared with DMSO ($*p < 0.05$).

[e] The type 3 immunostimulation of aloe-emodin was compared with DMSO ($*p < 0.05$).

Table 5
Anthraquinones modulated the secretion of IL-10 and IFN-γ[a]

Drug	Dose	Index (test/medium control)	
		IL-10	IFN-γ
Medium		1.00 ± 0.12[b]	1.00 ± 0.09[c]
DMSO	0.10%	1.15 ± 0.03	1.37 ± 0.13
PHA	5 μg/ml	1.15 ± 0.03	62.9 ± 4.19*
Aloe-emodin	10 μg/ml	0.83 ± 0.17	0.64 ± 0.13
	1 μg/ml	1.41 ± 0.27*	1.61 ± 0.12*
Rhein	10 μg/ml	0.87 ± 0.21	0.73 ± 0.02

[a] The drug effects were expressed as index (test/medium control). ANOVA and multiple comparison were used to evaluate the difference between test drug and medium control ($*p < 0.05$).

[b] 141 ± 16.9 pg/ml.

[c] 8.6 ± 0.12 pg/ml.

4. Discussion

Several studies have indicated that emodin can suppress the proliferation of mitogen-activated lymphocytes (Huang et al., 1992; Kuo, Meng, & Tsai, 2001b) and chrysophanol exhibits anti-allergic actions (kim et al., 2000). However, the results from this study showed that four anthraquinones, including emodin and chrysophanol, exhibited significant immunostimulating effects on the resting lymphocytes. The discrepancy in results between the previous reports and this study could be due to the different study methods. Results from previous reports showed that emodin suppressed the mitogen-activated lymphocytes (Huang, Chu, & Lee Chao, 1991; Huang et al., 1992), but it directly stimulated the proliferation of resting PBMC and/or the secretion of IFN-γ, without mitogen activation, which was used in this study. These different results indicated that emodin might be a dual immunomodulator, which could enhance or suppress immune response dependent on the activated state of immune cells. IFN-γ acts as a multipotent immune activator because it plays an important role in modulating nearly all phases of immune and inflammatory responses and is associated with effective host defense against intracellular pathogens and cancer (Billiau & Vandenbroeck, 2001). In this study, the concomitance of activated lymphocytes and secretion of IFN-γ was only in agreement with the results of positive controls (PHA, ConA). There were three types of immunostimulation after treatment with anthraquinones according to different chemical structure and concentration. For instance, chrysophanol significantly stimulated the proliferation of resting PBMC, whereas emodin exhibited the same response at high dose, but augmented secretion of IFN-γ at a low dose, indicating the R1 moiety and dose as the key points for different responses between chrysophanol and emodin. Similar variations were also noted when comparing aloe-emodin, chrysophanol and rhein, with the R2 moiety and dose as the key factors for this phenomenon (Table 2). Lymphocyte proliferation was correlated to the increase of the number of $CD8^+$ T cells and activated PBMC in mitogen PHA and to the elevation of the number of $CD4^+$ T cells in anthraquinones groups. Augmentation of IFN-γ secretion might be mainly due to the $CD8^+$ T cells because the secretion was significantly elevated in PHA but not in anthraquinone groups. The secretion of IFN-γ by $CD8^+$ T cells was in accordance with the observation made by Sad et al., despite the different species tested (Sad, Marcotte, & Mosmann, 1995). However, slight elevation of IFN-γ and IL-10 secretion after treatment with aloe-emodin might be correlated to the activated $CD4^+$ T cells. In conclusion, four anthraquinones were found to possess immunomodulatory activity to stimulate proliferation of resting PBMC and to secret multipotent cytokines

which differed from potent mitogen PHA, and the responsive patterns were dependent on concentration and chemical structure. The property of augmenting resting PBMC proliferation and secretion of multipotent immunomodulators IFN-γ and IL-10 by anthraquinones may partly explain the reason for the popular use of some anthraquinones-containing medicinal plants such as *C. tora* and *P. cuspidatum* for treating infections and inflammatory diseases (Hatano, Uebayashi, Ito, Shiota, & Tsuchida, 1999; Huang, 1993; Maity et al., 1998; Spring, 1989).

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