DOI:10.1021/jf103581r

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Five Bitter Compounds Display Different Anti-inflammatory Effects through Modulating Cytokine Secretion Using Mouse Primary Splenocytes in Vitro

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Bitter foods are generally recognized as anti-inflammatory agents in traditional Chinese medicine. To verify the anti-inflammatory effects of some bitter compounds in foods or plants, five bitter compounds, aloperine, amygdalin, berberine, crotaline, and naringenin, were selected and added to primary mouse splenocytes in the absence or presence of lipopolysaccharide (LPS) under four different in vitro experimental models. Anti-inflammatory cytokine secretions such as interleukin (IL)-10 and pro-inflammatory cytokines such as IL-6 as well as tumor necrosis factor (TNF)-α were determined using enzyme-linked immunosorbent assay (ELISA). The results showed that all selected bitter compounds except amygdalin exhibited apparent cytotoxic effects. On the basis of changes in the secretion profiles between anti- and pro-inflammatory cytokines, the five selected bitter compounds demonstrated anti-inflammatory activities via modulating either IL-6/IL-10 or TNF-α/IL-10 ratios at noncytotoxic doses. Berberine and naringenin treatments showed the strongest potential for anti-inflammation among the five selected bitter compounds. Berberine especially displayed strong anti-inflammatory activity in both preventive and repair manners.

KEYWORDS: Bitter compounds; aloperine; amygdalin; berberine; crotaline; interleukin (IL)-6; IL-10; naringenin; primary splenocytes; tumor necrosis factor (TNF)-α

INTRODUCTION

Inflammation, which is a pattern of response to injury (*1*), involves the local accumulation of fluid, plasma proteins, and white blood cells resulting from physical damage, infection, or a local immune response (*2*). However, uncontrolled inflammation causes the overproduction of inflammatory mediators such as pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF-α) (*3*) or inhibits the secretion of anti-inflammatory cytokine by immune effector cells, such as IL-10 (*4*). It may further result in a variety of chronic inflammation derived diseases such as cardiovascular disease, cancer, diabetes, migraine, and arthritis (*1*). Luckily, some natural products exhibiting anti-inflammatory activity, such as aspirin and aspirin-like drugs including the nonsteroidal anti-inflammatory drugs (NSAIDs), have been widely used to alleviate inflammation damage such as fever, pain, migraine, and arthritis (*1*). Terpenoids, flavonoids, allied phenolic and polyphenolic compounds, and sulfur-containing compounds are now accepted as providing an anti-inflammatory function (*5*). Among these phytochemicals, some terpenoids such as andrographolide and amarogentin, flavonoids, and allied phenolic and polyphenolic compounds such as naringenin, caffeic acid, chlorogenic acid, resveratrol, and sulfur-containing compounds such as phenylthiocarbamide have a bitter taste to humans and are classified as bitter compounds (*6*).

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In traditional Chinese medicine, bitter foods have been recognized as anti-inflammation agents for centuries. Recently we found that bitter melon juice exhibited prophylactic effects on lipopolysaccharide (LPS)-induced inflammation of murine peritoneal macrophages (*7*). However, the effectiveness of bitter compounds against inflammation remains unclear. Five bitter compounds, aloperine, amygdalin, berberine, crotaline, and naringenin, were selected according to published papers and added in vitro to mouse primary splenocytes in the absence or presence of LPS to evaluate their anti-inflammatory effects.

Bitter compounds may exist in foods or plants and have different physiological functions. Aloperine is one of the quinolizidine alkaloids in *Sophora alopecuroides* L. (*8*), possibly having anti-inflammatory, antipyretic, anticancer, antiviral. and antinociceptive effects (*9*). Amygdalin (D-mandelonitrile-β-gentiobioside) in apricot seeds is one of the cyanogenic glycosides, possibly having antinociceptive and anti-inflammatory effects (*10*, *11*). Berberine is one of the isoquinolines and may exist in many herbal plants, such as *Hydrastis canadensis* (goldenseal), *Rhizoma coptidis* (Huanglian), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric) (*12*). In Chinese pharmacopoeia, *R. coptidis* (Huanglian) and *B. vulgaris* (barberry) are classified as heat-removing vegetable drugs. Crotaline is one of the pyrrolizidine alkaloids and may exist in *Crotolaria spectabilis*, possibly having an anticancer activity (*13*). Naringenin (4′,5,7-trihydroxyflavanone) is one of the flavonoids abundant in grapefruit and tomato, possibly having anticancer, antimutagenic, and antiatherogenic activities (*14*−*17*). Although some bitter compounds seem to

 Published on Web 12/14/2010

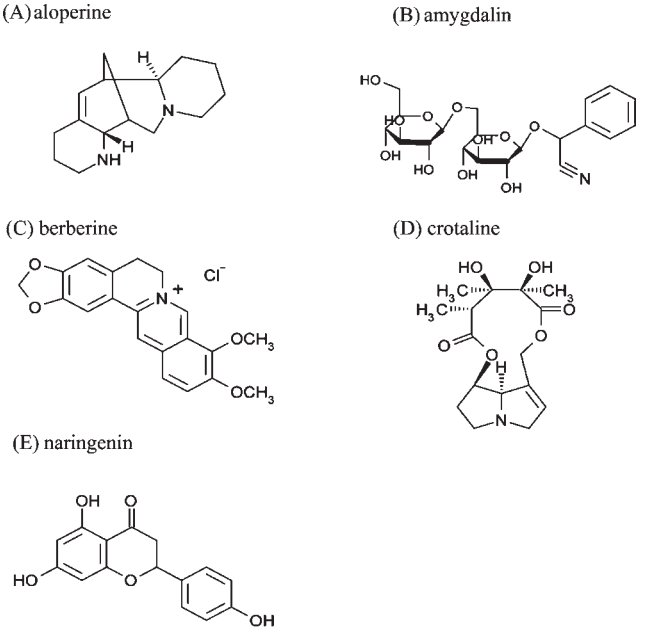

Figure 1. Chemical structures of aloperine (**A**), amygdalin (**B**), berberine (**C**), crotaline (**D**), and naringenin (**E**).

have differential physiological functions, scientific evidence for bitter compounds selected in this study against inflammation is still limited.

We hypothesize that the selected bitter compounds are potentially valuable as anti-inflammatory agents. This study selected five bitter compounds, including aloperine, amygdalin, berberine, crotaline, and naringenin, to evaluate their anti-inflammatory activities using mouse primary splenocytes in the absence or presence of LPS under four different in vitro experimental models. Anti-inflammatory cytokine secretions such as IL-10 and proinflammatory cytokines such as IL-6 as well as TNF-α were determined. On the basis of changes in the secretion profiles between anti- and pro-inflammatory cytokines, the anti-inflammatory activities of five selected bitter compounds were judged.

MATERIALS AND METHODS

Selected Bitter Compounds. Five bitter compounds, aloperine (Sigma-Aldrich, St. Louis, MO), amygdalin (Sigma-Fluka, Munich, Germany), berberine chloride (Sigma, Bangalore, India), crotaline (Sigma, Bangalore, India), and naringenin (Sigma, U.K.) were purchased at the highest available purity. The purities of aloperine, amygdalin, berberine, crotaline, and naringenin were 96, ≥97, ≥95, ≥95, and ~95%, respectively. The chemical structures of aloperine, amygdalin, berberine, crotaline, and naringenin are shown in **Figure 1**.

Preparation of Selected Bitter Compounds. Aloperine was dissolved in dimethyl sulfoxide (DMSO; Wako, Osaka, Japan) to prepare a stock solution at the indicated concentration of 215 mM and sterilized using a filter (Millipore, Bedford, MA) with 0.20 μm pore size. Amygdalin was dissolved in tissue culture medium (TCM) to prepare a stock solution at the indicated concentration of 220 mM and sterilized using a filter (Sartorious, Goettingen, Germany) with 0.20 μm pore size. Berberine was dissolved in DMSO to prepare a stock solution at the indicated concentration of 135 mM and sterilized using a filter (Millipore) with 0.20 μm pore size. Crotaline was dissolved in DMSO to prepare a stock solution at the indicated concentration of 155 mM and sterilized using a filter (Millipore) with 0.20 μm pore size. Naringenin was dissolved in DMSO to prepare a stock solution at the indicated concentration of 370 mM and sterilized using a filter (Millipore) with 0.20 μm pore size. All stock solutions were stored at −80 °C for future use.

Primary Splenocyte Isolation. The animal use protocol listed below was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC. Female BALB/c mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, ROC, and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University, College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The mice were housed and kept on a chow diet (laboratory standard diet). After the mice were acclimatized for 2 weeks, the animals were weighed, anaesthetized with diethyl ether, and immediately bled using retro-orbital venous plexus puncture to collect blood. Immediately after blood collection, the animals were sacrificed using CO_2 inhalation for primary splenocyte culture studies. The splenocytes were prepared by aseptically removing the spleens from BALB/c mice. Spleens were homogenized in TCM (a defined commercial serum replacement; Celox Laboratories Inc., Lake Zurich, IL), a mixture of 10 mL of TCM, 500 mL of RPMI 1640 medium (Atlanta Biologicals, Inc., Norcross, GA), and 2.5 mL of antibiotic−antimycotic solution (100× PSA) containing 10000 units/mL of penicillin, 10000 μg/mL of streptomycin, and 25 μg/mL of amphotericin B in 0.85% saline (Atlanta Biologicals, Inc.). Single spleen cells were collected and treated by lysing the red blood cells with RBC lysis buffer [0.017 M Trizma base (Sigma-Aldrich Co., St. Louis, MO), 0.144 M ammonium chloride (Sigma-Aldrich Co.), pH 7.4, 0.2 μm filtered]. Splenocytes were isolated from each animal and adjusted to 1×10^7 cells/mL in TCM with a hemocytometer using the trypan blue dye exclusion method.

Determination of Cell Viability (MTT Assay). To evaluate the possible cytotoxic effects of bitter compounds, the cell viabilities of splenocytes treated with selected bitter compounds at different concentrations were determined by 3-(4,5-dimethylthiazol-2-diphenyl)-2,5-tetrazolium bromide (MTT) assay. All bitter compound stock solutions were aseptically diluted into working solutions using TCM before use. The splenocytes (50 μL/well) in the absence or presence of samples (50 μL/well) at different concentrations were cultured in 96-well plates and incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 72 h. Aliquots of 10 μL of 5 mg/mL MTT [(Sigma M5655, St. Louis, MO) in phosphate-buffered saline (PBS)] were added to each well in the 96-well plate. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for another 4 h. After incubation, the plates were centrifuged at 400*g* for 10 min. The culture medium was then discarded. The plates were carefully washed with PBS buffer three times. Aliquots of 100 μL of DMSO were added to each well and oscillated for 30 min to extract formed insoluble formazan. The absorbance (A) was measured at 550 nm on a plate reader (ELISA reader, ASYS Hitech, GmbH, Austria). The cell viability was described as the survival rate (%) compared to the mean absorbency of the control. The cell viability (%) in each biological determination was calculated using the following equation: cell viability (%) = $[(A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$. The noncytotoxic doses of bitter compounds were selected to conduct anti-inflammation assessments.

Anti-inflammation Assessments of Selected Bitter Compounds in Vitro. To verify anti-inflammatory effects of bitter compounds, five bitter compounds, aloperine, amygdalin, berberine, crotaline, and naringenin, were prepared and added to mouse primary splenocytes in the absence or presence of LPS under four different experimental models in vitro. Among the five selected bitter compounds, naringenin, which has been reported to have many physiological functions in vitro and in vivo, was chosen as a positive control because it is a bitter trihydroxyflavanone abundant in many foods.

Model A. To evaluate the effects of bitter compounds treated alone on immune cells, the splenocytes (0.50 mL/well) in the absence or presence of samples (0.50 mL/well) at different concentrations were cultured in 24-well plates and incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 48 h. An endotoxin and B-cell mitogen, LPS (Sigma-Aldrich Co., L-2654, St. Louis, MO) at a final concentration of 2.5 μg/mL in culture was selected as a positive control in each experiment. The plates were centrifuged at 400*g* for 10 min. The supernatants in the cell cultures were collected and stored at −80 °C for cytokine assays.

Model B. The endotoxin LPS were selected to stimulate the inflammatory mediator secretions by splenocytes in this model (*18*). An inflammation-concurrent cell culture model was designed using LPS addition and test samples together (*7*). The splenocytes (0.50 mL/well) were cocultured

with LPS (at a final concentration of 2.5 μg/mL in culture) and samples (0.50 mL/well in total) at different concentrations. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 48 h. The plates were centrifuged at 400*g* for 10 min. The supernatants in the cell cultures were collected and stored at −80 °C for cytokine assays.

Model C. To evaluate the prophylactic effects of selected bitter compounds on LPS-induced inflammation in vitro, the splenocytes were first cultured in the absence or presence of samples at different concentrations and incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 24 h. The plates were centrifuged at 400*g* for 10 min. The supernatants in the cell cultures were discarded to remove samples. The cultured cells in the plate were washed twice with 1 mL of sterile Hank's balanced salts solution (HBSS) containing a mixture of 50 mL of 10× HBSS buffer (Hyclone, SH30015.02, Logan, UT), 2.5 mL of 100× PSA, 20 mL of 3% bovine serum albumin (BSA, Sigma A9418, St. Louis, MO), 2 mL of 7.5% $NaHCO_3$ (Wako, 191-01305, Osaka, Japan), and 425.5 mL of water. To each well in the plate was added 1 mL of LPS at a final concentration of 2.5 μg/mL TCM medium, and the plate was incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for another 24 h. The plates were centrifuged at 400*g* for 10 min. The supernatants in cell cultures were collected and stored at −80 °C for cytokine assays.

Model D. To evaluate the repair effects of selected bitter compounds on LPS-induced inflammation in vitro, the splenocytes were first cultured in the absence or presence of LPS at a final concentration of 2.5 μg/mL TCM medium and incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 24 h. The plates were centrifuged at 400*g* for 10 min. The supernatants in the cell cultures were discarded to remove LPS. The cultured cells in the plate were washed twice with 1 mL of sterile HBSS. To each well in the plate was added 1 mL of sample at different concentrations, and the plate was incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for another 24 h. The plates were centrifuged at 400*g* for 10 min. The supernatants in cell cultures were collected and stored at −80 °C for cytokine assays.

Measurement of Pro-inflammatory and Anti-inflammatory Cytokine Levels Secreted by Splenocyte Cultures Using an Enzyme-Linked Immunosorbent Assay (ELISA). The splenocyte culture supernatants of four biological replicates ($n = 4$) in each individual treatment were collected to measure pro-inflammatory cytokine (IL-6 and TNF-α) and anti-inflammatory cytokine (IL-10) levels using sandwich ELISA kits, respectively. The IL-6, IL-10, and TNF-α concentrations were assayed according to the cytokine ELISA protocol from the manufacturer's instructions (mouse DuoSet ELISA Development system, R&D Systems, Minneapolis, MN). The sensitivity of these cytokine assays was 15.6 pg/mL.

Statistical Analysis. Values are expressed as mean ± SD of four biological determinations and analyzed statistically using one-way ANOVA followed using Duncan's new multiple-range test. Differences among treatments were considered to be statistically significant if $P < 0.05$. Statistical tests were performed using SPSS version 12.0.

RESULTS AND DISCUSSION

Effects of Selected Bitter Compounds Treated Alone on Mouse Primary Splenocytes Cell Viability. We first determined the possible cytotoxicity of the five selected bitter compounds by treating primary splenocytes with test samples at various concentrations for 72 h followed by a MTT assay. The remaining cell viability was determined by the MTT assay, showing that all selected bitter compounds have differential cytotoxicities on the splenocytes (**Figure 2**). The half-maximal (50%) inhibitory concentration (IC_{50}) was approximately measured on the basis of **Figure 2**. The IC_{50} values of aloperine, amygdalin, berberine, crotaline, and naringenin are 81.08, >2750, 6.6, 1230, and 230 μM, respectively. The magnitude of cytotoxicity on the splenocytes in order was berberine (IC_{50} = 6.6 μM) > aloperine (IC_{50} = 81.08 μM) > naringenin (IC_{50} = 230 μM) > crotaline (IC_{50} = 1230 μM) > amygdalin (IC_{50} > 2750 μM). To avoid excessive cytotoxicity at high doses, appropriate concentrations (much smaller than IC_{50} or not significantly different from the control) of selected bitter compounds were adopted to further assess their anti-inflammatory effects under different in vitro experimental models. The adopted concentrations of aloperine, amygdalin, berberine, crotaline, and naringenin were 10, 21, and 42 μM; 40, 170, and 700 μM; 0.8, 1.6, and 3.3 μM; 60, 120, and 250 μM; and 18, 35, and 70 μM, respectively. Compared to the controls, most of the adopted bitter compound concentrations did not significantly affect the cell viability of mouse primary splenocytes (**Figure 2**). Thus, the indicated noncytotoxic concentrations on immune cells were used for the following experiments.

Among the five selected bitter compounds, berberine treatment showed the strongest cytotoxic effects (IC_{50} = 6.6 μM) on mouse primary splenocytes. Berberine is an isoquinoline alkaloid. Orfila et al. reported that the strong cytotoxic effects of berberine result from its structure, which is similar to that of 8-hydroxy-7.8-dihydro derivatives (**Figure 1**) (*19*). To study the immunomodulatory effects of selected bitter compounds in vitro, cytotoxic doses should be avoided to prevent a side effect due to toxic cellular damage. In this study we adopted noncytotoxic concentrations of bitter compounds for in vitro anti-inflammation studies (**Figure 2**). This study also suggests that bitter compounds should be carefully used in vivo to avoid their cytotoxic effects.

Effects of Selected Bitter Compounds Treated Alone (Model A) on Cytokine Secretions on Mouse Primary Splenocytes. To examine the anti-inflammatory potential of bitter compounds on mouse primary splenocytes in vitro, selected bitter compounds at the indicated noncytotoxic concentrations were added to splenocyte cultures for 48 h. Pro-inflammatory cytokine secretions including TNF-α as well as IL-6 and an anti-inflammatory cytokine, IL-10, by splenocytes were determined. **Table 1** shows the effects of the five selected bitter compounds on the secretion of pro-inflammatory cytokines TNF-α as well as IL-6 and an anti-inflammatory cytokine, IL-10, by the splenocytes. The results showed that berberine and naringenin treatments at the indicated concentrations, respectively, significantly ($P < 0.05$) inhibited TNF-α secretions by the splenocytes in a dose-dependent manner (**Table 1**). Aloperine, berberine, and crotaline treatments at the indicated concentrations significantly ($P < 0.05$) inhibited IL-6 secretions by the splenocytes. Amygdalin treatment at 170 μM markedly ($P < 0.05$) inhibited IL-6 secretions. However, the five selected bitter compounds treated at the indicated concentrations did not significantly ($P > 0.05$) inhibit IL-10 secretions. The IL-10 secretion levels in the treated splenocyte cultures were lower than the sensitivity of the ELISA kit used in this study. The results from this study suggest that the five selected bitter compounds exhibited anti-inflammatory potential via inhibiting TNF-α or IL-6 secretions (**Table 1**). However, berberine treatment exhibited the best anti-inflammatory activity via decreasing both pro-inflammatory cytokine TNF-α and IL-6 secretions by the treated splenocytes.

Effects of Selected Bitter Compounds Cotreated with LPS (Model B) on Cytokine Secretions by Mouse Primary Splenocytes. To examine the anti-inflammatory potential of bitter compounds on mouse primary splenocytes in the presence of LPS, the five selected bitter compounds at the indicated noncytotoxic concentrations were added to LPS-stimulated splenocytes (model B) for 48 h. Secretions of pro-inflammatory cytokines including TNF-α as well as IL-6 and an anti-inflammatory cytokine, IL-10, by LPS-stimulated splenocytes were determined. **Table 2** shows the effects of the five selected bitter compounds on the secretion of pro-inflammatory cytokines TNF-α as well as IL-6 and an anti-inflammatory cytokine, IL-10, by LPS-stimulated splenocytes. The results showed that naringenin treatments at the indicated concentrations significantly ($P < 0.05$) inhibited TNF-α secretions by the LPS-stimulated splenocytes (**Table 2**). Aloperine and berberine treatments at the indicated concentrations significantly ($P < 0.05$) inhibited IL-6 secretions by the LPS-stimulated

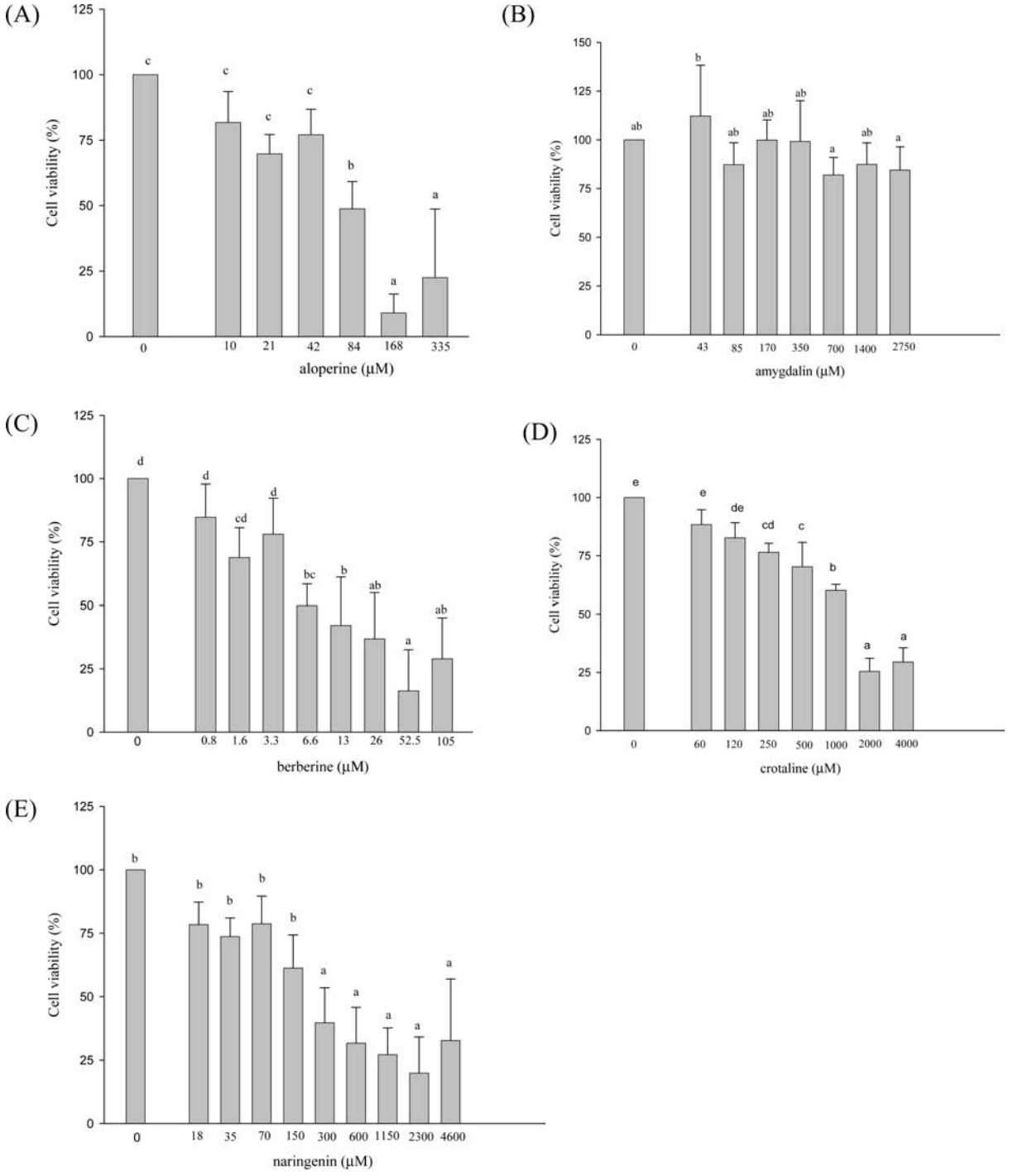

Figure 2. Effects of in vitro treatments of aloperine (**A**), amygdalin (**B**), berberine (**C**), crotaline (**D**), and naringenin (**E**) on cell viabilities using primary splenocytes from female BALB/c mice. Values are expressed as mean ± SD ($n = 4$ biological determinations) and analyzed using one-way ANOVA, followed by Duncan's new multiple-range test. Bars not sharing common letters are significantly different ($P < 0.05$) from each other. The original cell density was 5×10^6 cells/mL. Lipopolysaccharide (2.5 μg/mL) was selected as a positive control in each experiment.

splenocytes. However, aloperine treatment at the indicated concentration also significantly ($P < 0.05$) inhibited IL-10 secretions by the LPS-stimulated splenocytes. Further analysis on the secretion ratios of pro-/anti-inflammatory cytokines by the LPS-stimulated splenocytes exhibited that amygdalin, berberine, and naringenin treatments significantly decreased IL-6/IL-10 ratios, respectively (**Table 2**). Aloperine, berberine, and naringenin decreased TNF-α/IL-10 ratios. Our results suggest that berberine and naringenin have strong anti-inflammatory potential against LPS-stimulated inflammation via decreasing both IL-6/IL-10 and TNF-α/IL-10 secretion ratios (**Table 2**).

Effects of Selected Bitter Compounds on LPS-Stimulated Cytokine Secretions by Mouse Primary Splenocytes under a Specified Preventive Experimental Model C. To further unravel the anti-inflammatory mechanisms of the five selected bitter compounds on LPS-induced inflammation, the indicated noncytotoxic concentrations were added to splenocyte cultures before (model C) or after (model D) LPS stimulation. Samples treated for 24 h before LPS addition simulated a preventive experimental model for anti-inflammation. **Table 3** shows the effects of the five selected bitter compounds on secretion of pro-inflammatory cytokines TNF-α as well as IL-6, and an anti-inflammatory cytokine, IL-10, by

Table 1. Effects of Five Selected Bitter Compounds Treated Alone (Model A) on Cytokine Secretions by Mouse Primary Splenocytes

		cytokine secretion[a] (pg/mL)		
sample	concn (μM)	TNF-α	IL-6	IL-10
aloperine	0	74.6 ± 10.6	82.0 ± 65.5^{B}	ND
	10	82.9 ± 92.8	19.3 ± 8.23^{A}	ND
	21	8.99 ± 16.2	22.2 ± 22.2^{A}	ND
	42	25.8 ± 23.6	ND	ND
amygdalin	0	74.6 ± 10.6	82.0 ± 65.5^{B}	ND
	40	53.8 ± 52.1	47.8 ± 25.9^{AB}	ND
	170	32.4 ± 26.1	13.5 ± 15.3^{A}	ND
	700	38.7 ± 49.0	30.6 ± 16.3^{AB}	ND
berberine	0	74.6 ± 10.6^{B}	82.0 ± 65.5^{B}	ND
	0.8	14.5 ± 17.1^{A}	23.5 ± 11.8^{A}	ND
	1.6	ND	20.1 ± 19.0^{A}	ND
	3.3	ND	21.3 ± 14.8^{A}	ND
crotaline	0	74.6 ± 10.6	82.0 ± 65.5^{B}	ND
	60	49.0 ± 20.2	24.6 ± 12.9^{A}	ND
	120	26.0 ± 31.0	22.8 ± 8.34^{A}	ND
	250	48.0 ± 36.1	21.2 ± 20.0^{A}	ND
naringenin	0	74.6 ± 10.6^{B}	82.0 ± 65.5	ND
	18	29.7 ± 21.7^{A}	70.4 ± 36.8	ND
	35	11.1 ± 14.6^{A}	55.3 ± 21.0	ND
	70	11.2 ± 19.9^{A}	64.6 ± 38.5	ND

[a] Values are mean $\pm$ SD (n = 4 biological determinations). Data are analyzed using one-way ANOVA, followed by Duncan's new multiple-range test. Values within the same column and same sample not sharing a common superscript are significantly different ($P < 0.05$) from each other. ND, means not detectable. The sensitivity of the ELISA kits used in this study was about <15.6 pg/mL. The original cell density was 5×10^6 cells/mL.

LPS-stimulated splenocytes under experimental model C. The results showed that amygdalin and berberine treatments at the indicated appropriate concentrations significantly ($P < 0.05$) inhibited TNF-α secretions by the LPS-stimulated splenocytes (**Table 3**). Aloperine, berberine, crotaline, and naringenin treatments at the indicated concentrations significantly ($P < 0.05$) inhibited IL-6 secretions by the LPS-stimulated splenocytes (**Table 3**). However, all five selected bitter compounds did not significantly affect IL-10 secretions by the LPS-stimulated splenocytes (**Table 3**). Further analysis on the secretion ratios of pro-/anti-inflammatory cytokines by the LPS-stimulated splenocytes exhibited that all five selected bitter compounds at the indicated appropriate concentrations decreased IL-6/IL-10 ratios (**Table 3**). Berberine, crotaline, and naringenin, respectively, decreased TNF-α/IL-10 ratios. The results suggest that berberine, crotaline, and naringenin have strong anti-inflammatory potential against LPS-stimulated inflammation via decreasing both IL-6/IL-10 and TNF-α/IL-10 secretion ratios under a specified preventive experimental model C (**Table 3**). Aloperine and amygdalin treatments also demonstrated a moderate anti-inflammatory potential against LPS-stimulated inflammation via just decreasing IL-6/IL-10 secretion ratios under the specified preventive experimental model C (**Table 3**).

Effects of Selected Bitter Compounds on LPS-Stimulated Cytokine Secretions by Mouse Primary Splenocytes under Specified Repair Experimental Model D. To unravel their repair effects on anti-inflammation, the five selected bitter compounds at the indicated noncytotoxic concentrations were added to splenocyte cultures after LPS stimulation (model D). Samples treated for 24 h after LPS addition simulated a repair experimental model for anti-inflammation. **Table 4** shows the effects of the five selected bitter compounds on secretions of pro-inflammatory cytokines TNF-α as well as IL-6 and an anti-inflammatory cytokine, IL-10,

Table 2. Effects of Five Selected Bitter Compounds Cotreated with LPS (Model B) on Cytokine Secretions by Mouse Primary Splenocytes

		cytokine secretion[a] (pg/mL)			pro-/anti-inflammatory cytokine secretion ratio[a] (pg/pg)	
sample[b]	concn (μM)	TNF-α	IL-6	IL-10	IL-6/IL-10	TNF-α/IL-10
aloperine	0	730 ± 185	1032 ± 661^{B}	469 ± 141^{B}	2.04 ± 0.84	1.59 ± 0.29^{A}
	10	597 ± 163	587 ± 337^{AB}	334 ± 104^{AB}	1.65 ± 0.47	1.81 ± 0.18^{AB}
	21	544 ± 104	401 ± 221^{A}	246 ± 110^{A}	1.57 ± 0.21	2.44 ± 0.71^{ABC}
	42	545 ± 156	376 ± 223^{A}	229 ± 96.1^{A}	1.55 ± 0.35	2.49 ± 0.47^{BC}
amygdalin	0	730 ± 185	1033 ± 661	469 ± 141	2.04 ± 0.84^{B}	1.59 ± 0.29
	40	691 ± 148	521 ± 306	500 ± 128	0.98 ± 0.36^{A}	1.40 ± 0.17
	170	629 ± 191	436 ± 257	422 ± 132	0.97 ± 0.29^{A}	1.51 ± 0.21
	700	634 ± 171	443 ± 268	377 ± 109	1.10 ± 0.38^{A}	1.71 ± 0.26
berberine	0	730 ± 185	1033 ± 661^{B}	469 ± 141	2.04 ± 0.84^{B}	1.59 ± 0.29^{BC}
	0.8	602 ± 165	453 ± 267^{A}	487 ± 135	0.87 ± 0.31^{A}	1.25 ± 0.14^{A}
	1.6	520 ± 179	319 ± 188^{A}	409 ± 131	0.73 ± 0.23^{A}	1.27 ± 0.14^{A}
	3.3	497 ± 172	298 ± 180^{A}	380 ± 128	0.73 ± 0.23^{A}	1.31 ± 0.19^{AB}
crotaline	0	730 ± 185	1033 ± 661	469 ± 141	2.04 ± 0.84	1.59 ± 0.29
	60	747 ± 172	612 ± 349	425 ± 154	1.38 ± 0.44	1.84 ± 0.40
	120	661 ± 158	479 ± 290	339 ± 129	1.33 ± 0.42	2.03 ± 0.39
	250	604 ± 151	487 ± 312	301 ± 113	1.51 ± 0.55	2.08 ± 0.34
naringenin	0	730 ± 185^{B}	1033 ± 661	469 ± 141	2.04 ± 0.84^{B}	1.59 ± 0.29^{B}
	18	593 ± 147^{AB}	1103 ± 529	615 ± 163	1.72 ± 0.39^{A}	0.97 ± 0.14^{A}
	35	468 ± 149^{A}	848 ± 420	563 ± 193	1.46 ± 0.31^{A}	0.84 ± 0.13^{A}
	70	407 ± 109^{A}	694 ± 338	531 ± 135	1.25 ± 0.30^{A}	0.77 ± 0.11^{A}

[a] Values are mean $\pm$ SD (n = 4 biological determinations). Data are analyzed using one-way ANOVA, followed by Duncan's new multiple-range test. Values within the same column and same sample not sharing a common superscript are significantly different ($P < 0.05$) from each other. The original cell density was 5×10^6 cells/mL. The sensitivity of ELISA kits used in this study was about <15.6 pg/mL. [b] The isolated splenocytes were cocultured with lipopolysaccharide (2.5 μg/mL) and samples at different concentrations for 48 h.

Table 3. Effects of Five Selected Bitter Compound Treatments on Lipopolysaccharide-Stimulated Cytokine Secretions by Mouse Primary Splenocytes under Specified Preventive Experimental Model C

		cytokine secretion[a] (pg/mL)			pro-/anti-inflammatory cytokine secretion ratio[a] (pg/pg)	
sample[b]	concn (μM)	TNF-α	IL-6	IL-10	IL-6/IL-10	TNF-α/IL-10
aloperine	0	835 ± 184	166 ± 48.1^{B}	71.8 ± 33.2	2.53 ± 0.86^{B}	13.1 ± 5.66
	10	652 ± 157	102 ± 45.5^{A}	66.3 ± 31.2	1.57 ± 0.30^{A}	10.7 ± 3.17
	21	679 ± 156	88.7 ± 26.8^{A}	60.9 ± 25.1	1.55 ± 0.54^{A}	11.8 ± 2.28
	42	699 ± 181	107 ± 28.9^{A}	44.8 ± 16.6	2.76 ± 1.56^{AB}	16.8 ± 5.06
amygdalin	0	835 ± 184^{B}	166 ± 48.1	71.8 ± 33.2	2.53 ± 0.86^{B}	13.11 ± 5.66
	40	567 ± 129^{A}	115 ± 25.0	73.6 ± 15.0	1.57 ± 0.10^{A}	7.74 ± 0.92
	170	611 ± 169^{AB}	121 ± 41.3	81.2 ± 39.8	1.55 ± 0.20^{A}	8.03 ± 1.58
	700	637 ± 151^{AB}	139 ± 53.4	86.7 ± 16.8	1.57 ± 0.42^{A}	7.33 ± 0.64
berberine	0	835 ± 184^{B}	166 ± 48.1^{B}	71.8 ± 33.2	2.53 ± 0.86^{B}	13.1 ± 5.66^{B}
	0.8	565 ± 88.4^{A}	111 ± 48.0^{AB}	77.4 ± 24.4	1.41 ± 0.17^{A}	7.67 ± 1.60^{A}
	1.6	539 ± 95.9^{A}	86.5 ± 34.8^{A}	76.3 ± 23.4	1.13 ± 0.31^{A}	7.24 ± 0.76^{A}
	3.3	569 ± 126^{A}	80.4 ± 39.0^{A}	77.9 ± 15.8	1.00 ± 0.26^{A}	7.30 ± 0.43^{A}
crotaline	0	835 ± 184	166 ± 48.1^{B}	71.8 ± 33.2	2.53 ± 0.86^{B}	13.1 ± 5.66^{B}
	60	687 ± 181	102 ± 45.8^{A}	86.2 ± 28.0	1.21 ± 0.44^{A}	8.21 ± 1.53^{A}
	120	679 ± 193	95.7 ± 39.0^{A}	73.8 ± 25.1	1.29 ± 0.16^{A}	9.33 ± 0.71^{AB}
	250	685 ± 206	98.8 ± 42.7^{A}	84.0 ± 29.1	1.18 ± 0.26^{A}	8.37 ± 1.68^{A}
naringenin	0	835 ± 184	166 ± 48.1^{B}	71.8 ± 33.2	2.53 ± 0.86^{B}	13.1 ± 5.66^{B}
	18	706 ± 281	107 ± 56.3^{AB}	85.1 ± 28.5	1.20 ± 0.21^{A}	8.18 ± 0.57^{A}
	35	790 ± 317	84.7 ± 37.0^{A}	86.6 ± 38.2	0.98 ± 0.08^{A}	9.25 ± 0.49^{AB}
	70	696 ± 193	82.8 ± 43.1^{A}	88.0 ± 29.0	0.93 ± 0.26^{A}	8.11 ± 1.35^{A}

[a] Values are mean $\pm$ SD ($n = 4$ biological determinations). Data are analyzed using one-way ANOVA, followed by Duncan's new multiple-range test. Values within the same column and same sample not sharing a common superscript are significantly different ($P < 0.05$) from each other. The concentration of LPS was 2.5 μg/mL. The sensitivity of the ELISA kits used in this study was about <15.6 pg/mL. The original cell density was 5×10^6 cells/mL. [b] The preventive model is that isolated splenocytes were precultured with samples for 24 h, then washed out the samples, and finally added with lipopolysaccharide (LPS) for another 24 h.

by LPS-stimulated splenocytes under experimental model D. The results showed that both TNF-α and IL-6 levels secreted by the LPS-stimulated splenocytes were too low to be detectable. However, aloperine and berberine treatments significantly inhibited IL-10 levels secreted by the LPS-stimulated splenocytes (**Table 4**).

Undoubtedly, IL-10 is generally recognized as an anti-inflammatory cytokine and is produced in late-stage inflammation by immune effector cells to inhibit the synthesis of other cytokines (*4*). In contrast to anti-inflammatory cytokines such as IL-10, pro-inflammatory cytokines are produced in an early inflammation stage. It was found that the maximum secretion of pro-inflammatory cytokines occurs at 12−48 h when inflammation starts (*20*, *21*). In our previous study we also found that TNF-α and IL-1β are produced at the early stage of LPS-induced inflammation. Exogenous IL-10 administration could inhibit TNF-α and IL-1β secretions by LPS-stimulated peritoneal macrophages in vitro (*22*). Taken together, IL-10 is synthesized in the late inflammation stage to cope with excessive TNF-α, IL-1β, or other pro-inflammatory mediator secretion. In this experimental model D, LPS-treated media were washed out after the first 24 h of incubation. Therefore, TNF-α, IL-1β, or other pro-inflammatory mediators were also removed from the cultures. In the second 24 h of incubation, selected bitter compound treatments may further inhibit secretions of pro-inflammatory mediators such as TNF-α and IL-1β, which may induce IL-10 secretion. Therefore, inhibition of IL-10 levels in experimental model D suggests that the five selected bitter compounds have slight repair effects against anti-inflammation, especially aloperine and berberine (**Table 4**).

We designed four in vitro models to evaluate the anti-inflammatory effects of the selected bitter compounds using mouse primary splenocytes in the absence or presence of LPS. The results revealed that all five selected bitter compounds more or less exhibited anti-inflammatory activities. However, different bitter compounds seem to have different effects on anti-inflammation in the absence or presence of LPS. On the basis of changes in pro-inflammatory cytokine levels, anti-inflammatory cytokines, or pro-/anti-inflammatory cytokine secretion ratios, berberine treatment at the appropriate concentration demonstrated the strongest anti-inflammation potential in both preventive and repair manners (**Table 5**). Berberine was reported to have anti-inflammatory potential in both in vitro and in vivo studies (*23*−*25*). This study provides further scientific evidence. Among the five selected bitter compounds, naringenin treatment at appropriate concentrations exhibited the second greatest potential for anti-inflammation in a preventive manner (**Table 5**). Naringenin is also reported to have anti-inflammatory potential via inhibiting nitric oxide (NO), prostaglandin E_2 (PGE_2), and pro-inflammatory cytokine production in different cells (*26*−*28*). This study further provides additional evidence. However, the anti-inflammation mechanisms of berberine and naringenin remain to be further clarified. In this study naringenin, one of the flavonoids present in foods consumed daily such as fruits and vegetables, was chosen as positive control for its anti-inflammatory properties via immunomodulation (*29*). Interestingly, berberine overall exhibited better anti-inflammatory ability than naringenin in this in vitro study (**Table 5**). A berberine effect on inflammation in vivo is under investigation in our laboratory.

Naturally occurring bitter compounds widely exist in different plants, such as isocoumarin in carrots, diterpenoids in cucurbits, toxic alkaloids in bitter yam [*Dioscorea dumetorum* (Kunth) Pax], saponin and dioscin in *Dioscorea tokoro* Makino, tannins in yams and tea, and naringenin in grapefruit and tomatoes (*16*). Bitter compounds may exert different physiological functions. It was found that andrographolide, a diterpenoid compound from

Andrographis paniculata, displays an anti-inflammatory activity via inhibiting the activation of nuclear factor (NF)-κB, suppressing inducible nitric oxide synthase (iNOS) expression, preventing oxygen radical production by human neutrophils, and inhibiting cyclooxygenase (COX)-2 expression in human fibroblast cells (*30*). Certain phenolic compounds from different sources have been found to decrease inflammatory mediator production by human whole blood cultures (*31*). This study selected five bitter compounds on the basis of published papers and their specific structures to assess their anti-inflammatory effects. On the basis of their chemical structures (**Figure 1**), aloperine, amygdalin, berberine, and crotaline are alkaloids, but naringenin is a polyphenol. Although this study found that the five selected bitter compounds more or less have anti-inflammatory activities, there seems no evidence for a relationship between bitter taste and anti-inflammatory effects. Whereas herbs or plants containing many anti-inflammatory alkaloids and flavonoids with bitter taste have traditionally been consumed, those with bitter taste showing adverse effects including pro-inflammatory effects would have not been used. Some sweet compounds have also shown anti-inflammatory effects [e.g., compounds in kudzu (*32*) or licorice (*33*)].

Table 4. Effects of Five Selected Bitter Compound Treatments on Lipopolysaccharide-Stimulated Cytokine Secretions by Mouse Primary Splenocytes under Specified Repair Experimental Model D

		cytokine secretion[a] (pg/mL)		
sample[b]	concn (μM)	TNF-α	IL-6	IL-10
aloperine	0	ND	ND	100 ± 22.8^{C}
	10	ND	ND	106 ± 19.6^{C}
	21	ND	ND	87.3 ± 16.7^{BC}
	42	ND	ND	66.8 ± 14.4^{AB}
amygdalin	0	ND	ND	100 ± 22.8
	40	ND	ND	85.6 ± 14.2
	170	ND	ND	89.4 ± 30.0
	700	ND	ND	86.7 ± 32.7
berberine	0	ND	ND	100 ± 22.8^{B}
	0.8	ND	ND	82.8 ± 28.1^{AB}
	1.6	ND	ND	55.5 ± 18.3^{A}
	3.3	ND	ND	73.0 ± 7.25^{AB}
crotaline	0	ND	ND	100 ± 22.8
	60	ND	ND	90.0 ± 11.6
	120	ND	ND	76.0 ± 21.8
	250	ND	ND	72.2 ± 28.4
naringenin	0	ND	ND	100 ± 22.8
	18	ND	ND	104 ± 12.6
	35	ND	ND	91.8 ± 29.3
	70	ND	ND	95.2 ± 27.1

[a] Values are mean $\pm$ SD (n = 4 biological determinations). Data are analyzed using one-way ANOVA, followed by Duncan's new multiple range test. Values within the same column and same sample not sharing a common superscript are significantly different ($P < 0.05$) from each other. The concentration of LPS was 2.5 μg/mL. ND means not detectable. The sensitivity of the ELISA kits used in this study was about <15.6 pg/mL. The original cell density was 5×10^6 cells/mL. [b] The repair model is that splenocytes were prestimulated with lipopolysaccharide (LPS) for 24 h, then washed out LPS, and finally added with sample for another 24 h.

We hypothesized that the anti-inflammatory activities of tested compounds are related to their specific ring structures or functional groups. Levita et al. analyzed andrographolide (3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8*a*-dimethylene-1-naphthalenyl]ethylidine]dihydro-4-hydroxy-2(3*H*)-furanone), a diterpenoid compound that shows a potent anti-inflammatory activity via inhibiting the activation of NF-κB, from computational chemistry aspects (*30*). The DNA binding region of NF-κB has mainly positive potential; therefore, a specific inhibitor should have negative potential to gain an electrostatic complementarity (*30*). Structurally, adrographolide contains three hydrogen bond donors (H atoms in hydroxyl groups attached to C-3, C-19, and C-14), five hydrogen bond acceptors (O atoms in hydroxyl groups attached to C-3, C-19, C-14, carbonyl, and lactone), and log P value 2.9 (P is an octanol−water partition coefficient) (*30*). We inferred a chemical compound having a molecular mass of under 500 Da (range from 160 to 500 Da) to have not more than 5 hydrogen bond donors and not more than 10 hydrogen bond acceptors, to possess an appropriate hydrophobic property as well, and perhaps have potent anti-inflammatory potential (*30*). Berberine has five hydrogen bond acceptors (four oxygen atoms and one nitrogen atom), whereas naringenin has three hydrogen bond donors (H atoms in hydroxyl groups attached to benzene groups; phenolic groups) and five hydrogen bond acceptors (O atoms in phenolic groups and a specific ring structure) (**Figure 1**). According to the inference, naringenin may have a superior or similar ability to berberine in hydrogen bond formation; however, berberine may have a much more appropriate hydrophobic property than naringenin. Our results that berberine's anti-inflammatory ability is superior to that of naringenin (**Table 5**) conform to the inference. Experimentally, it is verified that some, but not all, phenolic compounds exhibit anti-inflammatory effects (*31*). Phenolic groups in phenolic compounds may contribute to anti-inflammatory effects via serving as hydrogen bond donors or acceptors; however, multiple factors including appropriate hydrophobic property, which involves an anti-inflammatory potential, should be included. Although amygdalin and crotaline have many hydrogen bond donors or acceptors, their comparatively low hydrophobic properties may decrease their anti-inflammatory effects. According to our preliminary results (data not shown), we conjectured that berberine may modulate cytokine expression via an alternate regulation of mRNA transcription (a transcriptional regulation) and mRNA stability (a post-transcriptional regulation) in immune cells. However, anti-inflammatory immunomodulation may be initiated from different pathway including extracellular or intracellular signaling. Unfortunately, some signaling pathways remain unknown; therefore, more data should be accumulated to clarify potential anti-inflammatory agents and their anti-inflammatory mechanisms.

Table 5. Summary of Five Selected Bitter Compound Treatments on Inflammation under Different in Vitro Experimental Models[a]

bitter compound	changes in levels of TNF-α or IL-6, model A	changes in IL-6/IL-10 or TNF-α/IL-10 ratios, model B	changes in IL-6/IL-10 or TNF-α/IL-10 ratios, model C	changes in levels of IL-10, model D
aloperine	+	−	+	+
amygdalin	+	+	+	
berberine	++	++	++	+
crotaline	+		++	
naringenin	+	++	++	

[a] ++, two anti-inflammation indicators; +, one anti-inflammation indicator; −, one inflammation indicator.

We have characterized mouse primary splenocytes in our previous studies. The cell constituents of primary splenocytes from BALB/c mice contained 41.54% of B cells and 47.11% of T cells, respectively (*34*). The spleen collects antigens from blood. Therefore, primary splenocyte cultures may directly reflect systemic immune responses. However, primary cultures from different individuals may cause a lot of experimental variation. Some results from this study showing a big variation resulted from individual diversity, not experimental error or sample variation.

In conclusion, this study selected five bitter compounds, aloperine, amygdalin, berberine, crotaline, and naringenin, to assess their anti-inflammatory activities using mouse primary splenocytes. All of the selected bitter compounds except amygdalin exhibited apparent cytotoxic effects. This study found that the five selected bitter compounds more or less have anti-inflammatory activities via modulating the secretion profiles of pro- and anti-inflammatory cytokines at noncytotoxic doses under four different experimental in vitro models. Berberine and naringenin treatments showed the strongest potential for anti-inflammation among the five selected bitter compounds. Berberine displayed particularly strong anti-inflammatory activity in both preventive and repair manners.

ABBREVIATIONS USED

IL-6, interleukin-6; IL-10, interleukin-10; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

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Received for review September 15, 2010. Revised manuscript received November 22, 2010. Accepted November 23, 2010. This study was kindly supported by research grant NSC98-2313-B-005-035-MY3 from the National Science Council, Taipei, Taiwan, ROC.