Food Sci. Biotechnol. Vol. 17, No. 6, pp. 1185 ~ 1190 (2008)

Food Science and Biotechnology

Immunostimulating and Anticancer Activities of Hot-water Extracts from *Acanthopanax senticosus* and *Glycyrrhiza uralensis*

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Abstract: When 10 kinds of herbal medicines were fractionated into hexane, MeOH, cold-water, and hot-water extracts, hot-water extracts from *Acanthopanax senticosus* (AS), *Glycyrrhiza uralensis* (GU), *Cichorium intybus* (CI), and *Polygonatum odoratum* (PO) showed the potent intestinal immune system modulating activity (1.72-, 1.62-, 1.60-, and 1.53-fold of control at 100 μg/mL, respectively). Especially, hot-water extracts from AS (215% compared with the control) and GU (187%) also had macrophages stimulating activity and mitogenic activity of splenocytes (7.1- and 6.5-fold) at 100 μg/mL. In addition, the effects of hot-water extracts from herbal medicines on anticancer activities were studied in mice. Hot-water extracts from AS and GU enhanced cytotoxicity of natural killer cell against cancer cell, Yac-1 (37 and 34% cytotoxicity) at E/T ratio 100:1, and colon 26-M3.1 cancer cell lines had significantly inhibited (82.1 and 75.2%) in experimental lung metastasis. These results suggest that hot-water extracts from *A. senticosus* and *G. uralensis* can be used as biological response modifiers to stimulate immune system and inhibit tumor.

Keywords: herbal medicine, hot-water extract, immunostimulating activity, anticancer activity

Introduction

Oriental herbal medicines generally take orally as the crude extracts which are obtained by the decoction of the mixture of several herbs (prescription). Crude extract contains not only low molecular weight substances such as alkaloids, saponins, and flavonoids, but also high molecular weight substances such as proteins, tannins, and polysaccharides (1). Although biologically active substances with low molecular weight in herbal medicines have been studied well, they can not account for all of the clinical effects achieved. During the last 20 years, a great number of immunostimulating macromolecular substances have been isolated from higher plants and fungi, and recently, several pharmacological activities have been also discovered in the polysaccharides isolated from traditional herbal medicines by several groups (2,3).

Acanthopanax senticosus has long been used prophylactically for various diseases such as chronic bronchitis, hypertension, and ischemia in East Asia. *A. senticosus* has also been known to be effective in reducing many types of stress and fatigues, and thus is called adaptogen (4,5). It has also been recently published that the systemic administration of the extracts of *A. senticosus* and its polysaccharide fraction have the potential to inhibit tumor metastasis through the activation of macrophage or natural killer (NK) cells as well as enhance the antigen-specific immune response (6).

Therefore, studies on bioactive high molecular weight materials, such as polysaccharides and proteoglycan, are important for elucidation of efficacy of oriental herbal medicines and development of new functional foods or medicines. The present paper deals with on bioactive plant macromolecules from Korean traditional herbal medicines in order to obtain the possibilities for functional foods and medical use.

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Received February 21, 2008; Revised May 23, 2008;
Accepted July 8, 2008

Materials and Methods

Materials Ten kinds of herbal medicines were obtained from the big mart (Cheongju, Korea). Voucher specimens of these plants were deposited at Division of Food and Biotechnology, Chungju National University in Jeungpyong, Korea (herbarium No. 001-010). RPMI-1640 medium and Hank's balanced salt solution (HBSS) for the cultivation of Peyer's patch and macrophage cells were obtained from Gibco-BRL Co. (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Cell Culture Laboratories (Cleveland, OH, USA), and penicillin, streptomycin, and amphotericin B from Flow Laboratories (Irvine, Scotland). Alamar Blue™ was obtained from Alamar Bio-Sciences Inc. (Sacramento, CA, USA).

Mice ICR and specific pathogen-free female Balb/c or C3H/He mice (5-7 weeks old) were purchased from Daihan-Biolink Co. (Chungbuk, Korea). The mice were housed and maintained at 24±1°C with constant humidity (55%). They had access to commercial chew pellet diet (Samyang Co., Ltd., Incheon, Korea), and water was freely available.

Preparation of various solvent fractions Dried herbal medicines (50 g) were homogenized by Ultra-Turrax® T-50 at 5,000 rpm for 20 min (Janke & Kunkel IKA-Labortechnik, Staufen, Germany). After filtration, the filtrate was separated (cold-water extract), and the residues were initially refluxed with hexane, followed by MeOH and further extraction with hot-water, in an increasing order of

their polarity. Each extract was centrifuged to remove insoluble materials (7,400×g, 30 min, 4°C), and was lyophilized into hexane, MeOH, cold-water, and hot-water extracts.

Assay of immunological activities ***Peyer's patch-mediated intestinal immune system modulation*****:** The activity was measured according to Hong *et al.* (7). Suspensions of Peyer's patch cells in RPMI 1640 medium supplemented with 5% FBS (RPMI 1640-FBS) were prepared from a small intestine of the C3H/He mice. An 180 μL of the cell suspension (2×10^6 cells/mL in RPMI 1640-FBS) was cultured with 20 μL of test sample in a 96-well flat bottom microtiter plate for 5 days at 37°C in a humidified atmosphere of 5% CO_2-95% air. Then, the resulting culture supernatant (50 μL) was incubated with bone marrow cell suspension (2.5×10^5 cells/mL) from C3H/He mice for 6 days in the same incubator. After 20 μL of Alamar Blue™ solution was added to each well, the cells were continuously cultured for 5-24 hr. The fluorescence intensity was measured to count the cell numbers with Fluoroskan II (Labsystems, Helsinki, Finland) at an excitation wavelength of 544 nm and emission wavelength of 590 nm during the cultivation according to Pagé *et al.* (8).

Macrophage stimulation**:** Three days before the experiment, the 6-week-old ICR mice were injected aseptically with 1 mL of 3% thioglycollate broth via the interperitoneal route. After cervical dislocation, peritoneal exudates cells were harvested by the injection of 5 mL of the cold RPMI 1640 medium containing 5 mM HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL). After the supernatant was discarded by the centrifugation (250×g, 10 min, 4°C), the cells were adjusted to 1×10^6 cells/mL in RPMI 1640 medium. The 0.2 mL of cell suspension was allowed to adhere onto the surface of the cell of a 96-well flat bottom microtiter plate for 2 hr, and non-adherent cells were removed by washing twice with RPMI 1640 medium-10% FBS (9). Macrophage-stimulating activity was measured by the procedure of Suzuki *et al.* (10) with slight modification. The adherent macrophage cells were cultured in the presence of test samples in a 96-well microplate for 24 hr. Macrophage in a 96-well microplate (1×10^5 cells/mL) was solubilized by the addition of 25 μL of 0.1% Triton X-100. One-hundred-fifty μL of 10.0 mM *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) was added to the reaction mixtures, and the absorbance at 405 nm was photometrically measured using a microplate reader (3550-UV; Bio-Rad Laboratories, Hercules, CA, USA).

Mitogenic response assay**:** For mitogenic response assay, splenic lymphocytes (2×10^5/well) from ICR mice were co-cultured with the indicated doses of samples in 96-well culture plates for 72 hr. Lymphocyte proliferation was assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT)-based colorimetric assay (11). Six hr prior to culture termination, 20 μL of MTT solution (5 mg/mL) was added to each cell and the cells were continuously incubated. Culture was terminated, and the medium was removed prior to adding dimethyl sulfoxide (DMSO) to dissolve formazan crystals formed in cells, and measured at 490 nm by a microtiter plate reader.

Tumor metastasis inhibition**:** Experimental lung metastasis of colon 26-M3.1 cells was assessed by intravenous (*i.v.*) inoculation of tumor cell lines into Balb/c mice (12). In the lung metastasis experiment, mice were given *i.v.* administration of samples (500 μg/mouse) 3 days after *i.v.* injections of 2.7×10^4 colon 26-M3.1/mouse. The mice were sacrificed 14 days after tumor inoculation, and their lungs were fixed in Bouin's solution. Lung tumor colonies were counted microscopically.

NK cell-mediated tumor cytotoxicity**:** Two Balb/c mice per group were intravenously administered their respective extract (100 μg/mouse), and their splenocytes were harvested after 1 day. Single splenocytes cell suspensions (100 μL/well) were added to ^{51}Cr-labelled Yac-1 cells (1×10^4 cells/100 μL/well) to obtain effector (splenocytes)-to-target (Yac-1) cell ratios (E/T ratio) of 100:1 in U-bottomed 96-well plates. The cultures were incubated for 6 hr at 37°C in 5% CO_2 air atmosphere. After incubation, the plates were centrifuged for 10 min at 900×g. The supernatant (100 μL) of each well was absorbed onto a cotton swab and monitored for radioactivity using a gamma counter (13). The percentage of cytotoxicity generated by the NK cells was calculated from the radioactivity (count/min) according to the following equation.

$$\text{Cytotoxicity (\%)} = [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$$

Statistical analysis The differences between the control (only saline without sample) and the treatment in the experiments were tested for statistical significance by the Student's *t*-test. A value of $p<0.05$ was considered to show that the test sample had statistically significant immunostimulating and anticancer activities.

Results and Discussion

Comparison of solvent extracts from herbal medicines on intestinal immune system modulating activity The dried and crushed herbal medicines subjected to various solvent extracts to confirm the immunostimulating activity were fractionated into hexane, MeOH, cold-water, and hot-water extracts. When each solvent extract was assayed on intestinal immune system modulating activity through Peyer's patch *in vitro*, hot-water extracts from *A. senticosus* (AS), *Glycyrrhiza uralensis* (GU), *Cichorium intybus* (CI), and *Polygonatum odoratum* (PO) showed the potent activity (1.72-, 1.62-, 1.60-, and 1.53-fold of the control at 100 μg/mL, Table 1). The other extracts, except cold-water extract from AS having intermediate activity (1.53-fold), showed little activity even at a high concentration (data not shown). This result suggested that hot-water extracts from AS, GU, CI, and PO enhanced the stimulatory response of intestinal immune system modulation. The gastrointestinal tract is presented with a potentially overwhelming antigenic load each day in the form of commensal bacteria and dietary antigens. The system must be able to discriminate pathogens that require a protective immune response from normal bacteria flora or food antigens, where developing and maintaining a state of nonresponsiveness is necessary (14). This characteristic of the mucosal immune response, known as oral tolerance, is not only an important homeostatic

Table 1. Intestinal immune system modulating activity of solvent extracts from herbal medicines

Plant name (100 μg/mL)	Fluorescence intensity of extracts			
	Cold-water	Hexane[1)]	MeOH	Hot-water
Control[2)]		470±30		
Acanthopanax senticosus (AS)	720±40*[3)]	390	570±110	810±40*
Glycyrrhiza uralensis (GU)	640±40*	520±70	530±80	760±50*
Cinnamomum cassia (CC)	500±70	400	480±60	580±110
Polygonatum odoratum (PO)	600±30*	350	490±130	720±40*
Origanum majorana (OM)	460	350	580±30*	500±30*
Thymus vulgaris (TV)	590±100	360	410	540±90
Cucuma longa (CL)	540±60	490±100	480±100	570±120
Artemisia vulgaris (AV)	630±150	440	560±120	650±30*
Syzygium aromaticum (SA)	630±30*	540±80	520±90	670±150
Cichorium intybus (CI)	640±40*	390	530±100	750±40*

[1)]Significant difference was not calculated because the values were lower than the control.
[2)]Only saline without sample.
[3)]*$p<0.05$, significant difference between the control and sample.

process but is being used as a therapeutic approach for certain autoimmune and inflammatory diseases (15). The intestinal immune system including Peyer's patch not only contributes to the defense system of the mucosa but also regulates systemic inflammation, resulting in suppression of allergic reactions and autoimmune diseases (16). Since the functional foods supplemented by hot-water extracts from herbal medicines, such as AS, GU, CI, and PO, are eaten, there is a possibility that these active herbal hot-water extracts will express its clinical effects through the intestinal immune system.

Immunostimulating activities of hot-water extracts from herbal medicines The effects of hot-water extracts from AS, GU, PO, and CI to stimulate the macrophages were also investigated *in vitro*. When the macrophage activation by hot-water extracts from herbal medicines was measured, hot-water extract from AS and GU showed the high macrophage activation (215 and 187% compared with the control), especially, hot-water extract from AS as potent as the positive control, lipopolysaccharide (LPS, Fig. 1), and the others also showed intermediate activities (137-153%, higher than the control) at a concentration 100 μg/mL. These results suggested that hot-water extracts of AS, GU, CI, and PO enhanced the stimulatory responses of macrophages. Processes that remove invading microbes and harmful foreign or endogenous substances are essential to maintain normal development and homeostasis in multicellular organisms (17). Phagocytic leukocytes play multiple roles in these immune processes, serving as a link between the innate and acquired immune systems and contributing to the inflammatory response, angiogenesis, and the promotion of wound healing (18). Importantly, phagocytes can directly kill invading microorganisms and tumor cells, using both oxidative and non-oxidative mechanisms (19). Thus the development of novel therapeutics to non-specifically augment macrophage immune responses represents an ideal strategy for enhancing defense against microbial infection (20). To investigate the macrophage activation by hot-water extracts from herbal medicines, hot-water extracts were examined by cellular lysosomal

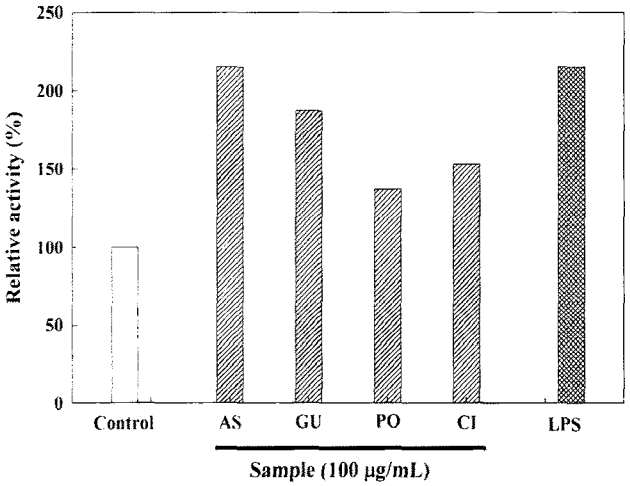

Fig. 1. Macrophage stimulating activity of hot-water extracts from medicinal plants. Control; only saline without sample. ▨; Hot-water extracts from herbal medicines (AS, *Acanthopanax senticosus*; GU, *Glycyrrhiza uralensis*; PO, *Polygonatum odoratum*; CI, *Cichorium intybus*). LPS, lipopolysaccharide; positive control (10 μg/mL).

enzyme activity and it was shown that more than 150% relative activity was detected in the hot-water extracts from AS, GU, and CI. This result shows that these herbal medicines important in the killing of microorganism in innate and adaptive immune response, and should represent an immunopotentiator and biological response modifiers.

Many biological substances have mitogenic activity and this property is useful for their practical applications. The mitogenic activity of hot-water extracts from AS, GU, PO, and CI was measured by the MTT-formazan method using spleen cells *in vitro*, which were isolated from ICR mouse without separation of T- and B-cells. As shown in Fig. 2, hot-water extracts from AS and GU also had mitogenic activity (7.1- and 6.5-fold of control at 100 μg/mL). Although mitogenic activities of lectins usually decreased at high concentrations (ca. 100 μg/mL) and showed maximum at the concentration of 5-10 μg/mL (21), AS and GU exhibited

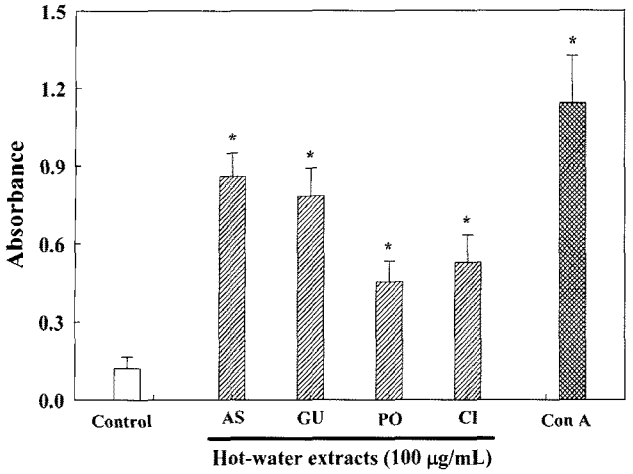

Fig. 2. Mitogenic effect of hot-water extracts from medicinal plants. Con A (Concanabalin A); positive control (10 μg/mL). *$p<0.05$; Significant difference between the control and sample. ▨; Hot-water extracts from herbal medicines (AS, *Acanthopanax senticosus*; GU, *Glycyrrhiza uralensis*; PO, *Polygonatum odoratum*; CI, *Cichorium intybus*).

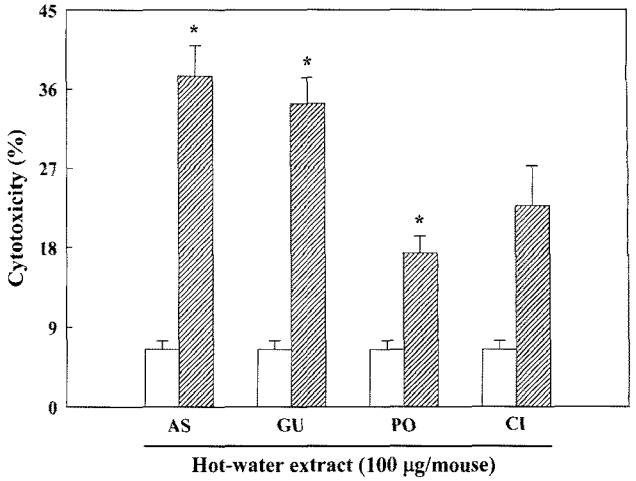

Fig. 3. NK cell-mediated tumor cytotoxicity of hot-water extracts from medicinal plants. □; Control (only saline without sample), ▨; Hot-water extracts from herbal medicines (AS, *Acanthopanax senticosus*; GU, *Glycyrrhiza uralensis*; PO, *Polygonatum odoratum*; CI, *Cichorium intybus*). *$p<0.05$, significant difference between the tumor control and sample.

such an increase to up to 100 μg/mL in this study (data not shown). However, investigation will be required to clarify the optimum concentration and cell specificity.

In the present study, hot-water extracts from AS, GU, CI, and PO have the immunostimulating activities, such as intestinal immune system modulating, macrophage activation, and mitogenic activity *in vitro*. These results would be assumed that these hot-water extracts enhance the immune system, but, the detailed mechanism and immunostimulating substances of their immune modulation effects are not fully. Therefore, further studies on these active substances for immunostimulation will provide us with important information, especially the relationship between the active substances and immune modulation.

Anticancer activities of hot-water extracts from herbal medicines The ability of hot-water extracts from herbal medicines to enhance NK cell activity was estimated by cytotoxicity against Yac-1, a NK-sensitive cell line, in 6 hr ^{51}Cr-release assay. As seen in Fig. 3, splenocytes of mice administered intravenously with 100 μg of hot-water extracts from AS and GU showed the higher NK-cell activity (37 and 34% cytotoxicity, E/T ratio 100:1) than those of CI and PO (21 and 16%), and these increased activity was also observed in a E/T ratio-dependent manner (data not shown). Since NK-cells were shown to play an important role in suppression of tumor growth and inhibition of tumor metastasis (22), hot-water extracts of AS and GU are able to enhance the natural immunity against tumors, and consequently inhibit tumor metastasis. The relevant effectors responsible for natural immunity against tumors have been identified as NK cells, lymphokine activated killer (LAK) cells and macrophages (23). Thus it is possible that the functional activation of NK, LAK, or macrophages results in the suppression of tumors and inhibition of tumor metastasis. Indeed, it had been shown that the activation of NK cells led to a reduction in metastatic colonication of tumors (24).

Table 2. Anti-metastasis effect of hot-water extracts from medicinal plants

Hot-water extract[1)]	Number of lung metastasis of colon 26-M3.1	
	Means±SD	Inhibition (%)
Tumor control[2)]	145±22	0
AS	26±4*[3)]	82.1
GU	36±10	75.2
PO	79±11*	45.5
CI	90±13*	37.9

[1)]The final concentration of sample was 500 μg/mouse (*i.v.* administration); AS, *Acanthopanax senticosus*; GU, *Glycyrrhiza uralensis*; PO, *Polygonatum odoratum*; CI, *Cichorium intybus*.
[2)]Tumor-beared one without sample administration.
[3)]*$p<0.05$, significant difference between the tumor control and sample.

In order to investigate whether hot-water extracts from herbal medicines inhibited tumor metastasis, the prophylactic effects of hot-water extracts were examined on the experimental lung metastasis, which was induced by colon 26-M3.1 cells. Table 2 shows that *i.v.* administration of hot-water extracts from AS, GU, PO, and CI 3 days before tumor inoculation similarly inhibited lung metastasis of colon 26-M3.1 cells (82.1, 75.2, 45.5, and 37.9% inhibition, respectively). This suggests that hot-water extracts from AS and GU are able to induce a prophylactic effect against lung metastasis induced by colon 26-M3.1 tumor cells, and water-soluble parts of hot-water extracts are one of the components related with this anti-tumor activity. Considering that the functional foods supplemented with the active herbal extracts enhances natural immunity, such as NK cell activity, and leads to the suppression of tumors, it might also augment the host defense system against tumors.

Many efforts have been made to develop and improve immunotherapy strategies for the treatment of malignancies. The use of biological response modifiers (BRMs) for enhancing host defense responses against tumors is one of

the most attractive alternatives to cytotoxic drugs (25). Activation of the innate immune system protects against foreign antigens, including tumors. Stimulation of the innate immune system has been attempted with a number of strategies, including cytokines constituents isolated from microorganisms and herbal plants, synthetic adjuvants, and oligonucleotides (26-29), and some have reached the clinical trials. The primary mechanism of immunomodulation by BRMs is activation of macrophages or NK cells (26-28), which can then lyse or inhibit the growth of tumor cells. Indeed, many experimental studies and clinical trials showed the natural immunity played an important role in blocking metastasis from primary tumors (30). NK cells and macrophages are responsible for the natural immunity against tumors (31). In addition, various cytokines, such as interleukin (IL)-12, tumor necrosis factor (TNF)-α, or IL-1β from macrophages, augment NK cell responses, and these proinflammatory cytokines can induce activation of adaptive immunity in part through stimulation of interferon (IFN)-γ production from NK cells (32).

A wide range of bioactive polysaccharides have been isolated from various medicinal plants, and these polysaccharides have been shown to possess immunomodulatory activity through their ability to modulate macrophage function (33). Indeed, botanical polysaccharides have been reported to increase macrophage cytotoxicity against tumor cells and microorganisms, activate phagocytosis, increase reactive oxygen species (ROS) and nitric oxide (NO) production, and enhance secretion of a variety of cytokines (33). Appropriate enhancement of these innate immune functions by bioactive compounds can then lead to improved host defense responsiveness (20). Moreover, most plant-derived polysaccharides are relatively non-toxic and do not cause severe side effects, which is a major problem associated with immunomodulatory bacterial polysaccharides and synthetic compounds (20). Thus, plant polysaccharides represent ideal candidates for therapeutics with immunomodulatory, anti-tumor, and wound-healing action.

From these results, hot-water extracts from herbal medicines enhances the stimulatory responses of immune system, and anti-cancer system such as NK-cell activation and metastasis suppression. For the evaluations on the effects *in vitro* and *ex vivo,* we need a more detailed understanding of the factors that enable hot-water extracts to play a role in these effects. Therefore more research on the characteristics of hot-water extracts and the biological mechanism of the effects *in vivo* are warranted.

Acknowledgments

This work was supported by research grant from the Small and Medium Business Administration, Korea. We express our sincere gratitude for the support.

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