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The Immunomodulating Effects of Aster Scaber THUNB Extracts in Mice

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***Aster scaber* THUNB (*AST* ; Charm-chui), a potent herbal medicinal plant, has a long tradition of use, being harvested as a wild plant, is said to stimulate appetite, and may act as a diuretic, antifebrile agent and painkiller. This study was conducted to investigate the immunomodulative effects of *AST* in mice, using in vitro and in vivo experiments. The immunomodulative effects were studied in vitro by measuring the proliferation of mice splenocytes and the production of three kinds of cytokines (IL-1β, IL-6, and TNF-α) by mice peritoneal macrophages which were cultured with sequential fractions of *AST* methanol extract (methanol, hexane, chloroform, ethylacetate, butanol and water). In an in vivo experiment using mice, different concentrations of *AST* water extract were orally administrated every other day for two weeks. The production of cytokines (IL-1β, IL-6, and TNF-α) secreted by activated macrophages, and the proliferation of mice splenocytes, were used as indices for immunocompetence. In vitro supplementation using six fractions of *AST* in the range of 1 to 100μg/ml enhanced splenocyte proliferation by 10.5% to 53% compared to the control. IL-1β production was significantly increased with the supplementation of butanol and water extracts of *AST*. Higher levels of IL-6 and TNF-α production were detected with supplementation of methanol, ethylacetate, butanol, or water extracts at the concentration of 100ug/ml. In the in vivo study, the highest proliferation of splenocytes was seen in the mice orally administrated with the AST water extract at the concentration of 500mg/kg body weight. In the case of cytokine production, there were no significant differences in the production of IL-1β and IL-6 among the treated groups and the control. However, TNF-α released by activated peritoneal macrophages were augmented by the oral administration of *AST* water extract. These results indicate that *AST* may enhance the immune functions by regulating splenocyte proliferation and cytokine production capacity in mice.**

Key words : splenocytes proliferation, macrophage, cytokine, immunomodulative effect

INTRODUCTION

Natural products are increasingly appreciated as leads for drug discovery and development. Studies of natural products have shown that they can have not only nutritional effects but also beneficial properties in curing various diseases and maintaining good health.[1),2)] Especially, those medicinal plants with long traditions of use have been shown to promote beneficial effects, such as enhancement of phagocytosis, cytokine induction, antibody production, induction of the mitogenic activity of spleen cells, anti-tumor activities,[3)] and antioxidant effects. Recently, many investigations have initiated searches for immunomodulating substances from natural food sources.[4)-10)] The immune system is a remarkably adaptive defense system that has evolved in vertebrates to protect them from invading pathogenic microorganisms and cancer. The immune system is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders. These cells and molecules act together against the foreign invaders by proliferation, differentiation and activation, and these alterations are dependent on environmental conditions.[11)] Immunomodulators are substances that enhance immune responses by regulating one or more components of the immune system. Some investigators have reported that plant isolates such as polysaccharides, lentinan, schizofilan, polysaccharide K (PSK) and ginsan, have immunoactivating properties.[4)-10)]

Aster scaber THUNB is a perennial plant which is widely cultivated as a culinary vegetable in Korea.[12)] Aster species have been used in traditional Chinese medicine to treat bruises, snake bites, headaches and dizziness.[13)] Recently, many studies have focused on the constituents of AST, including triterpene glycosides, saponins, monoterpene, and volatile compounds.[14)-18)] Lee et al reported that powdered *AST* decreases lipid peroxidation, and that Mn-SOD activity increased in the liver of rats which had been subjected to alcohol-induced oxidative stress.[19)] Quinic acid derivatives of AST were proposed as a therapeutic agent in Alzheimers disease because of their neuroprotective effects.[20)] Kwon et al

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reported that four quinic acid derivatives from *AST* showed inhibitory activities against the human immunodeficiency virus-1 (HIV-1).[21] However, very little research has been conducted on the immunomodulative effects of *AST*. Therefore, this study was performed to evaluate the immunomodulative effect of *Aster scaber* T_{HUNB} extracts in mice.

MATERIALS AND METHODS

1. Animals

Six-week-old ICR male mice (30±2g) were purchased from the KFDA (Korean Food and Drug Administration), and were kept under standardized animal house conditions (temperature 22±2℃; photoperiod approximately 12 hours of light and 12 hours of dark daily; and relative humidity at 50 - 60%). Pelleted food and tap water were available ad libitum. Prior to the experiment, at least 7 days were allowed for the mice to become acclimatized to animal house conditions and daily handling.

2. Chemicals

RPMI medium 1640, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from GIBCO BRL (Grand Island, N.K., USA). Streptomycin-penicillin, lipopolysaccharide (LPS), sodium bicarbonate, ammonium chloride, trisma base, trisma hydrochloride, trypan blue solution, 3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT), and DMSO (Dimethylsulfoxide) were purchased from the Sigma Chemical Co., St. Louis, USA. Thioglycollate was obtained from the DIFCO Lab. (Detroit, MI, USA). Cytokine kits were obtained from the Intergen Co., USA. Solvents used for extraction were extra pure grades.

3. Preparation of *AST* extracts

Fresh *Aster scaber* T_{HUNB} was purchased from the Kyungdong oriental market in Seoul, washed, dried, and powdered. Three consecutive decoctions were taken from two hundred grams of *AST* powder, in a boiling water bath with 600ml of methanol or distilled water under reflux for 3 hours. Some of the methanol extracts were subjected to sequential fractionations with hexane, chloroform, ethylacetate, and then n-butanol (Fig 1). The methanol, hexane, chloroform, ethylacetate, and n-butanol fractions, and an aqueous layer remaining after the n-butanol fractionation, were independently evaporated under reduced pressure at 60℃, and completely dried by lyophilization. The dried methanol extracts and each of the dried solvent fractions were dissolved in RPMI 1640 containing 10% FBS and less than 0.01 % dimethyl sulfoxide as a final concentration, and used as samples for the in vitro assay. The water extracts were also evaporated and dried like the other fractions, and dissolved in filtered distilled water for use in the in vivo assay.

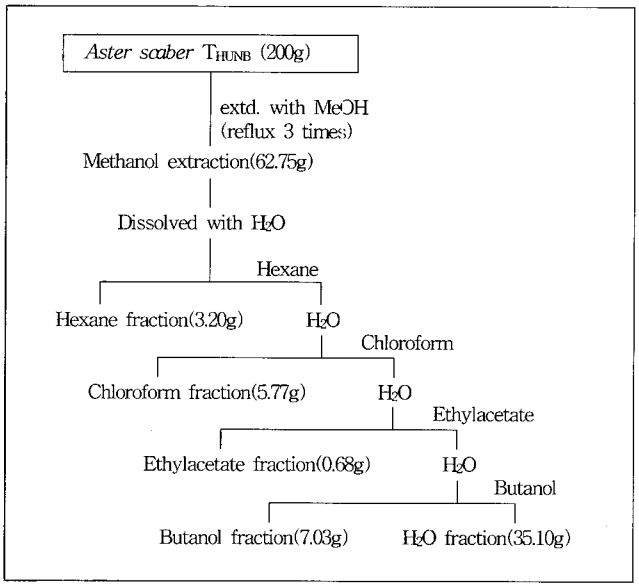

Fig 1. Schematic diagram for solvent fractions from methanol extract of Aster scaber T_{HUNB}.

4. *AST* extract treatment

In the in vitro experiment, 6 fractions of *AST* extracts - in concentrations of 1㎍/㎖, 10㎍/㎖, 50㎍/㎖, 100㎍/㎖, and 250㎍/㎖ - were added to the culture of splenocytes and peritoneal macrophages, and cultured for 48 hours.

For the in vivo experiments, doses of *AST* water extracts in concentrations of 0, 20, 100, 500 and 1000 mg/kg body weight were administered orally every other day for two weeks to five groups of mice, with 6 mice in each group. Mice were sacrificed on the 15th day after the beginning of the experiment, and splenocytes and peritoneal macrophages were collected for culture. Cells were cultured for 48 hours with or without mitogens (ConA or LPS).

5. Splenocytes preparation

Splenocytes were prepared by Mishells method.[22] Spleen was aseptically removed from the ICR mice sacrificed by cervical dislocation. Using the flat end of a sterile syringe plunger, spleen was gently crushed in an ice-cold RPMI 1640. A subsequent cell suspension was placed in a centrifuge tube through a 200-㎛ mesh nylon screen. The cell suspension was left aside for 10 minutes to precipitate the unbroken cell debris. The upper part of the cell suspension was carefully transferred to a new centrifugal tube and centrifuged at 400×g for 10 minutes. The resuspended pellet of spleen cells

was again suspended in lysing buffer to remove red blood cells, and then incubated for 5 minutes at room temperature with occasional shaking. A washing medium was added to fill the tubes, and the tubes were then centrifuged at 400×g for 10 minutes; the supernatant was then discarded. The pellet was washed again and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium containing 10% FBS, 100U/ml penicillin and 100㎍/ml streptomycin.

6. Spleen index

After the spleen was aseptically removed, it was weighed and the spleen index was calculated to standardize the differences in body weights of the mice[23]:

$$\text{Spleen index} = \sqrt{\text{weight of spleen(g)} \times 100 / \text{weight of mouse(g)}}$$

7. Splenocyte proliferation assay

Cell proliferations were determined by MTT colorimetric analysis, which is reported to be similar to the results of the 3H-thymidine uptake assay (which is frequently used for cell cytotoxicity assays).[24)-26)] In the in vitro experiment, the same volumes of prepared splenocytes suspensions of 5×10^6 cells/ml were plated in 96-well plates (Corning Glass, Corning, USA). Meanwhile, *AST* extracts (at different final concentrations of 0, 1, 10, 50, 100, and 250㎍/㎖), ConA (5㎍/㎖) or LPS (15㎍/㎖) were also added. The cells in 96-well plates (5×10^6 cells/ml) were exposed to various concentrations of extracts and were incubated at 37℃, and at 5 % CO_2 in air, for 48 hrs. In the in vivo experiment, splenocytes of every group were separately suspended to 5×10^6 cells/ml and plated in 96-well plates. ConA (5㎍/㎖) or LPS(15㎍/㎖) was added as a positive control and incubated as in the in vitro experiment.

After 44 hours of incubation, 10㎕ MTT solution (5mg/ml of phosphate buffered saline) was added and further incubated for 4 hrs at 37℃. After aspirating the supernatant from the wells, 150㎕ of DMSO (Dimethylsulfoxide; Sigma) was added for the dissolution of formazan crystals. The optical density (OD) of the samples was monitored by an ELISA reader at a wavelength of 540nm. MTT (tetrazolium salt) is cleaved only by metabolically active cells and is reduced to a colored formazan, and the color (OD value) reflects cell viability quantitatively. Proliferation of splenocytes was calculated by the following equation:

$$\text{Proliferation}(\%) = (\text{OD of sample} / \text{OD of control 1}) \times 100$$

8. Primary culture of macrophages

Thioglycollate was intraperitoneally injected into ICR mice at a rate of 2 ml of 4 % thioglycollate per 30g of body weight. Macrophages were collected from peritoneal lavages of the mice 3 days after injection, washed twice with cold RPMI medium 1640 (L-glutamine, 25 mM HEPES buffer, and sodium bicarbonate), and resuspended in RPMI containing 10 % FBS. Macrophages in the suspension were stained with trypan blue, their numbers counted by using a microscope, and then diluted to 1×10^6 cells/ml with the same medium. The diluted macrophages were seeded into 24-well culture plates (Corning, USA, 1ml/well) and incubated at 37 ℃ under 5 % CO_2. After preincubation for 2 hours, the medium was replaced with fresh medium to remove the non- adherent cells, and then various concentrations (1, 10, and 100㎍/ml) of extracts were added. In the in vivo experiment, cells from every group were incubated only with or without mitogen.

9. Measurement of cytokine (IL-1 β, IL-6, TNF- α) production in mouse peritoneal macrophages

Cells were incubated at 37℃ under 5% CO2 conditions for 48 hours, and then the supernatant medium was collected for the measurement of cytokines. Cytokine concentration was determined by using the ELISA method. Briefly, culture supernatant was added to 96-well ELISA plates pre-coated with goat anti-rabbit antibodies (Intergen Co., USA). It was then incubated with polyclonal anti-murine IL-1β, IL-6 or TNF-α antibody. After binding of biotinylate cytokine conjugate, streptavidin-conjugated alkaline phosphate and substrate solution were added. Optical density was measured at 540nm in the ELISA plate reader within 5 minutes of adding the amplifier solution.

10. Statistics

All values are expressed as mean ± SD from 6 observations. The Students t-tests for unpaired observations between the control and experimental samples were carried out for statistical evaluation of the differences; p values of 0.05 or less were considered as statistically significant. All statistical analyses were performed using the SAS system.

RESULTS AND DISCUSSION

1. Mitogenic activity of *AST* extracts on spleen cells

In the in vitro experiment, we examined the mitogenic activity of AST on splenocyte proliferation in the presence of six extracts of AST by MTT assay. In this assay, ConA (Concanavalin A), a common T cell mitogen, was added at the level of 5㎍/ml, or LPS (Lipopolysaccharide), a B cell mitogen4), was added at a level of 15㎍/ml, and these were used as the positive controls. The splenocyte proliferation with either ConA or LPS was

Table 1. Proliferation of splenocyte cultured with six different fractions of *AST* extracts

Conc.(μg/ml) / fraction	Proliferation(%)[1] 10	50	100	250	500	1000
Methanol	16.80±8.75[2]	32.06±3.65	52.99±0.15	34.28±11.30	-26.13±1.53	-67.58±0.95
Hexane	13.76±1.68	23.25±1.53	31.59±5.03	41.13±11.66	28.14±0.15	-62.42± 21.36
Chloroform	24.54±7.73	38.87±2.19	-5.36±14.29	-88.97±0.29	-86.55±0.36	-85.31±0.80
Ethylacetate	13.87±4.30	15.77±10.06	26.19±1.31	-9.43±7.80	-69.07±0.29	-80.88±0.656
Butanol	10.46±1.39	15.00±4.88	32.68±3.94	51.70±10.28	-75.98±4.52	-68.45±1.46
Water	11.55±0.73	16.55±4.16	20.31±2.19	5.46±0.58	-7.84±5.98	-33.51± 2.62
ConA	78.19±3.01					
LPS	49.85±2.01					

1) Proliferation(%) = (mean of O.D. in test wells / mean of O.D. in control wells -1) ×100
2) The data present the mean values ± S.D., n=6

increased to 78.19±3.01% and 49.85±2.01%. The chloroform fractions of AST enhanced splenocyte proliferation at lower concentrations of the extracts examined (24.54±7.73% at 10μg/ml; 38.87±2.19% at 50μg/ml). With 100μg/ml of methanol extract, splenocyte proliferation was increased to 52.99±0.15%, and this level was higher than those of the LPS stimulated group as shown in Fig 2. Supplementation of high concentrations (250μg /ml) of chloroform and ethylacetate fractions suppressed splenocyte proliferation, but the methanol, hexane, butanol and water fraction supplementations enhanced splenocyte proliferation. Lee reported that splenocyte proliferation was enhanced by the supplementation of some plant extract at low concentrations (10 and 50μg/ml), but that splenocyte proliferation was suppressed when samples were treated with high concentrations (250μg/ml) of plant extract.[27]

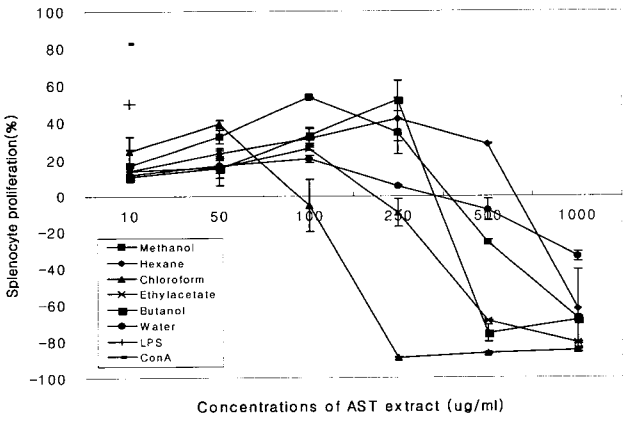

Fig 2. Proliferation of mice splenocytes cultured with Aster scaber T_{HUNB} extracts in six fractions.Spleen cells (5 × 106 cells/ well) were cultured with fraction of the extract on 96-well plate for 48 h. After culture, the degree of splenocyte proliferation was measured by MTT assay.

The data present the mean values±S.D., n=3

2. Cytokine production by macrophages treated with *AST* in vitro.

Normal peritoneal macrophages collected from ICR mice were cultured with 0, 10, and 100μg/ml of *AST* extracts for 48 hours. LPS (15μg/ml) treatments were used for comparative purposes. Cytokine levels in the culture supernatant were measured by the ELISA method. Macrophages are activated in a non-specific manner when antigens of infectious agents invade the body, and may secrete various cytokines (IL-1, Il-6, IL-12, TNF, IFN, etc) and chemicals (NO, prostaglandins, etc) for communication to other immune cells, killing the invading pathogens and inducing fundamental host defense systems. Cytokines such as IL-1, IL-2, IL-6 and TNF are frequently tested in relation to nutrition, and IL-1β, IL-6 and TNF-α are the main cytokines secreted by activated macrophages.[28)-31)] IL-1β is a representative cytokine which is secreted after activation of macrophages in the initial phase.[32] IL-1β concentration in the control group was 186.54±10.38pg/ml, while it was significantly increased to 210.48±23.45pg/ml, 191.05± 10.43pg/ml and 213.36±12.96pg/ml in the group of *AST* butanol (100μg/mℓ) and water fractions (10 and 100 μg/ mℓ), respectively. Koji reported that supplementation of celosian (from the seed of Celosia argentea, 10μg/ml) significantly activated IL-1β production and that this activity was concentration-dependent.[33] Compared to the control level of 282.88±24.78pg/ml, the addition of the extracts of methanol, ethylacetate, butanol or water fractions at a concentration of 100μg/ml significantly increased levels of IL-6 production (370.00±65.00pg/ml, 560.00±50.00pg/ml, 769.17±22.68pg/ml, and 647.50± 12.50pg/ml, respectively). Lee reported that higher levels of IL-6 production were observed when the methanol, chloroform, or water fractions of pine needles were added at a concentration of 100μg/ml.[27] There were significant differences in the production of TNF-α among

the treated groups and the control (36.69±9.90pg/ml), and the addition of 100㎍/ml of methanol, ethylacetate, butanol, or the water fraction increased the production of TNF-α (87.76±3.11pg/ml, 83.82±5.40pg/ml, 81.93± 8.53 pg/ml, and 86.10±3.94pg/ml, respectively). Studies of the immunomodulating effects of polysaccharides extracted from Ganodema lucieum show significant improvements in the production of TNF-α.[34] Koji reported that celosian significantly induced TNF-α production in mice, and suggested a possible immunoactivating function for celosian from this result.[33] Meanwhile, quercetin, one of the bio-flavonoids which is contained in onion and garlic, suppressed TNF-α production by mice macrophages.[35] Our in vitro experiment showed that butanol and water fractions increased IL-1β production, and chloroform and water fractions increased IL-6 production.(Fig 3-5)

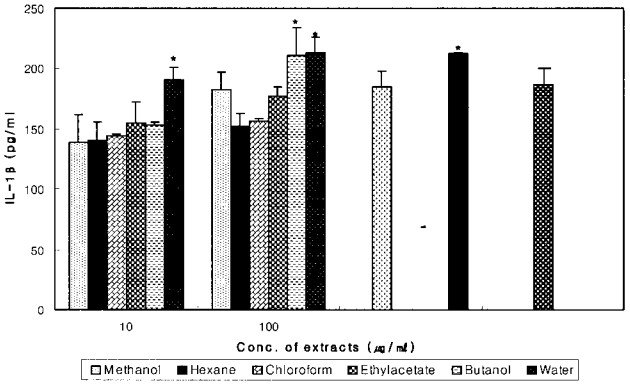

Fig 3. IL-1β production by mice peritoneal macrophage cultured with six different fraction of Aster scaber T_{HUNB} extracts.

The data present the mean values ± S.D., n=3. *Significantly different at p<0.05.

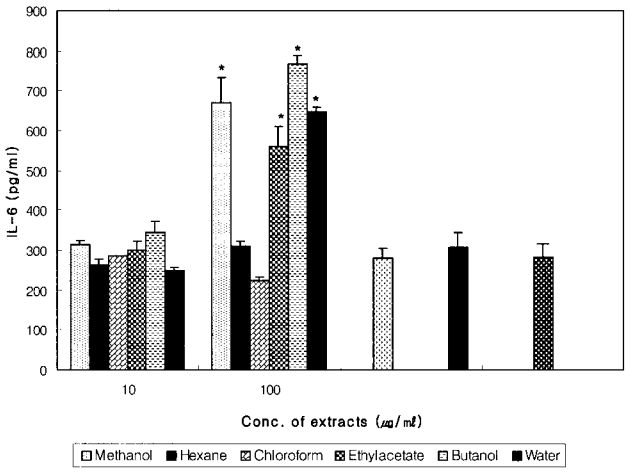

Fig 4. IL-6 production by mice peritoneal macrophage cultured with six different fraction of Aster scaber T_{HUNB} extracts.

The data present the mean values ± S.D., n=3. *Significantly different at p<0.05.

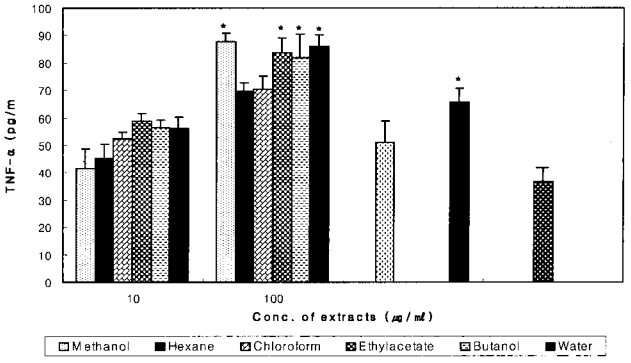

Fig 5. TNF-α production by mice peritoneal macrophage cultured with six different fraction of Aster scaber T_{HUNB} extracts.

The data present the mean values ± S.D., n=3. *Significantly different at p<0.05.

3. Oral administration of *AST* and splenocyte proliferation

Many studies using blood or tissue from experimental animals are currently in progress by researchers who are interested in the various effects of food intake on the body.[36)-37)] However, regarding immune systems, the spleen is a major organ, and the size or the proliferation of splenocytes can be an indicator of immune function.[38)-39)] instead of using blood or tissue. Oral administration of *AST* water extracts in this study didnt enhance splenocyte proliferation in all administered groups, except for the 20mg/kg b.w. (body weight) group without mitogenic stimulation, as shown in Fig 6. However, splenocyte proliferation was enhanced in the group supplemented with 500 mg/kg b.w. of AST extract, following treatment with ConA or LPS. In a previous study on C. Cristata L., splenocyte proliferation in the plant administered groups was enhanced, compared to the control groups, when they were treated with ConA or LPS.[40]

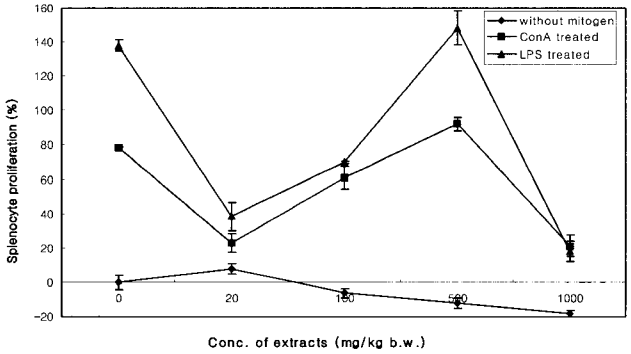

Fig 6. Increase in proliferaiton of splenocytes of mice orally administered with different levels of Aster scaber T_{HUNB} water extract treated with or without mitogen. Spleen cells (5 × 106 cells/well) were cultured with or without mitogen on 96-well plate for 48 h. After culture, the degree of splenocyte proliferation was measured by MTT assay.

The data present the mean values ± S.D. of 6 mice.

4. Oral administration of *AST* and cytokine production

Immune responses are regulated by many cytokines secreted by T-cells and macrophages. Macrophages are known to play an important role in host defense mechanisms. When macrophages are stimulated with bacterial products, a variety of cytokines and chemicals are released to induce fundamental defense mechanisms. The activated macrophages can preferentially lyse tumor cells and also secrete the cytokine tumor necrosis factor which can kill tumor cells. Macrophages also show increased phagocytosis against target cells, including tumor cells. Polysaccharides from plant materials have antitumor, immunomodulating, anti-inflammatory, hypoglycemic and antiviral activities.[41)-44)] In the present in vivo experiment, eight-week-old mice were fed ad libitum on a chow diet and the different concentrations of *AST* extract were orally administrated every other day for 2 weeks. The production of cytokines (IL-1β, IL-6, and TNF-α) secreted by activated macrophages in the supernatant, cultured with/without LPS (lipopolysaccharide) or ConA (Concanavalin A), was used as an index for immunocompetence (fig 7 - 9). Oral supplementation of *AST* water extracts didnt enhance the production of IL-1β and IL-6 without any mitogenic stimulation, but the levels of IL-1 β and IL-6 were slightly enhanced at a concentration of 500mg/kg b.w. of AST extract with LPS treatment (175.37±3.94pg/ml and 175.37±3.94pg/ml, respectively), compared to those levels of IL-1β and IL-6 in control groups (168.45±5.23pg/ml and 168.45±5.23 pg/ml, respectively). In LPS-stimulated animals, the group supplemented with 500mg/kg b.w. of AST extract showed significantly higher concentrations of TNF-α (746.70±36.21pg/ml) compared to the control (603.59± 16.16pg/ml). In the 100mg/kg b.w. supplementation group, TNF-α production, at (632.27±15.00pg/ml), was enhanced compared to the control (603.59±16.16pg/ml), but this was not statistically significant. Oral administration of many substances derived from plant materials, including Xanthii strumariu, Bezoar Bovis, Shi-ka-ron, pine needles, and C. cristata, were also reported to enhance cytokine production by mice macrophages.[27),40),45)] These results may support the possibility of polysaccharides from plant material, including *AST* water extracts, acting as immunomodulators,.

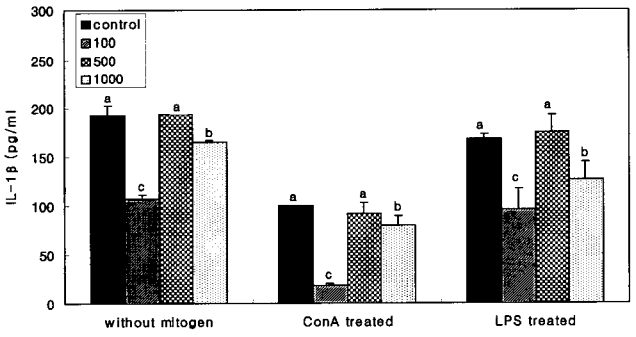

Fig 7. IL-1β production by activated peritoneal macrophage of mice orally administered with different levels of Aster scaber T_{HUNB} water extract treated with or without mitogen.

The data present the mean values ± S.D. n=6 The different letters within every mitogen groups are significantly different from each other at α =0.05 as determined by Duncan's multiple range test (a>b>c).

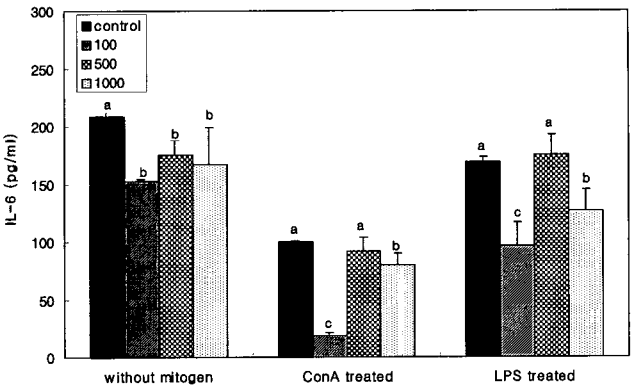

Fig 8. IL-6 production by activated peritoneal macrophage of mice orally administered with different levels of Aster scaber T_{HUNB} water extract treated with or without mitogen.

The data present the mean values ± S.D. n=6 The different letters within every mitogen groups are significantly different from each other at α =0.05 as determined by Duncan's multiple range test (a>b>c).

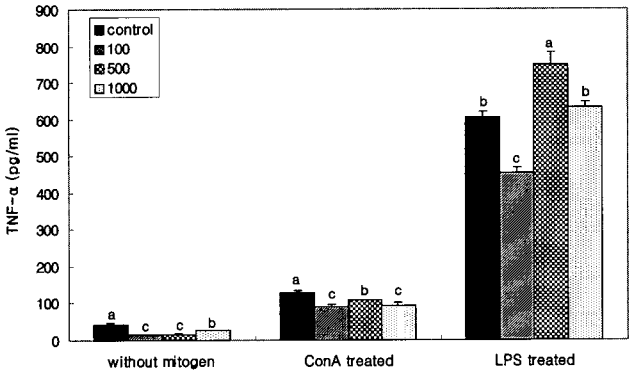

Fig 9. TNF-α production by activated peritoneal macrophage of mice orally administered with different levels of Aster scaber T_{HUNB} water extract treated with or without mitogen.

The data present the mean values ± S.D. n=6 The different letters within every mitogen groups are significantly different from each other at α =0.05 as determined by Duncan's multiple range test (a>b>c).

5. Conclusion

This study was performed to evaluate the potential of *Aster scaber* THNUB as an immunomodulator. In an in vitro experiment, the production capacity of cytokines (IL-1β, IL-6, and TNF-α) was used as an immunocompetence index; the cytokines were secreted by activated mouse macrophages (1×106 cells/ml), which were cultu-

red with three concentrations (10 and 100μg/ml) of methanol extracts of *AST* and 5 fractions (hexane, chloroform, ethylacetate, butanol, and water) obtained from methanol extract. Mouse splenocyte proliferation in the presence of various extracts from *AST* was also examined by MTT assay. The production of IL-1β was significantly enhanced with the addition of water extracts in all two(10 and 100μg/ml), and butanol extracts at the concentration of 100μg/ml. Compared to the control, higher levels of IL-6 production were detected when methanol, ethylacetate, butanol, or water fractions were added at a concentration of 100μg/ml. There were significant differences in the production of TNF-α between some treated groups (methanol, ethylacetate, butanol, and water fractions) and the control. Observations of mouse splencocyte proliferation indicated that supplementation using six fractions of *AST* in the range of 1 to 100μg/ml enhanced splenocyte proliferation by 10.5 to 53%, compared to the control. In vitro supplementation of a chloroform fraction of *AST*, in the range of 10 - 50μg/ml, enhanced proliferation by 25 - 39% compared to that of the control.

In the in vivo study, mice were orally administered for two weeks with water extracts of *AST*, and this enhanced the immune function in similar fashion to the in vitro experiment. Splenocyte proliferation was increased in the group supplemented with 500 mg/kg b.w. of AST extract. The animals in groups supplemented with *AST* extract produced lower levels of all three cytokines without any mitogen stimulation, compared to cytokine levels of the control group. In the LPS-stimulated animals, slightly higher concentrations of IL-1β and IL-6 were detected in the group supplemented with 500 mg/kg b.w. of AST extract. TNF-α production was significantly enhanced at the 500 mg/kg b.w. supplementation level. Therefore, it could be suggested that water extracts of *Aster scaber* T_{HUNB} may regulate immune functions by enhancing in vivo cytokine production when peritoneal macrophages are activated. Also, the activation of mouse peritoneal macrophages with LPS appeared to be effective in increasing the production of cytokine. The results of this study suggest that *AST* may contain immunomodulative agents, which are soluble in the polar solvents. Further investigations are needed to identify the stimulative components, the mechanism by which the immunomodulating activity may exert its effect, and the clinical effects of *AST* supplementation.

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