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Pharmacological Activities of a New Glycosaminoglycan, Acharan Sulfate Isolated from the Giant African Snail *Achatina fulica*

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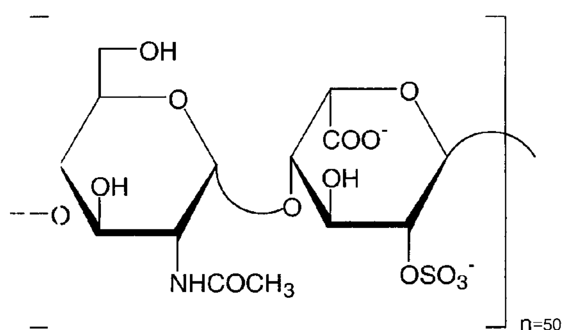
Acharan sulfate (AS) is a glycosaminoglycan (GAG) prepared from the giant African snail, *Achatina fulica*. In this study, some biological activities of AS were evaluated on the basis of structural similarities to heparin/heparan sulfate and the biological functions of GAGs. We demonstrated that it exhibited strong immunostimulating activities as measured by carbon clearance test in mice and *in vivo* phagocytosis. It also exhibited a significant hypoglycemic activity in epinephrine (EP)-induced hyperglycemia as well as antifatigue effects by weight-loaded forced swimming test. And it showed hypolipidemic activities in cholesterol-rich mixture induced hyperlipidemia in rats. The above results indicate that AS has diverse biological activities and suggest therapeutically important target molecules.

Key words: *Achatina fulica*, Acharan sulfate, Immunomoulator, Hypoglycemic, Antifatigue, Hypolipidemic

INTRODUCTION

Acharan sulfate (AS) is a glycosaminoglycan recently isolated from the giant African snail, *Achatina fulica* (Kim *et al.*, 1996; Jung *et al.*, 2001). This polysaccharide has an uncomplicated repeating disaccharide structure of → 4)-α-D-2-acetamido 2-deoxy-glucopyranose (1 → 4)-α-L-idopyranosyluronic acid-2-sulfate (1 → which is structurally related to heparin and heparin sulfate. (Linhardt and Toida, 1997), but is distinctly different from known members of these classes of glycosaminoglycans.



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Accordingly, AS, as a novel bioactive polysaccharide from *Achatina fulica*, was subjected to evaluate its biological activities. In this paper, we examined some pharmacological activities of AS and we demonstrated that it exhibited a significant hypoglycemic activity in EP-induced hyperglycemia, a significant immuno-stimulating as well as hypolipidemic activities in mice.

MATERIALS AND METHODS

Materials

AS (MW Ca.100,000) was isolated and purified from *Achatina fulica* as previously described (Kim *et al.*, 1996). Epinephrine (EP), glycogen, α-tocopherol acetate, an zymosan were purchased from Sigma Chem., Co. (St. Louis, MO, USA). Glucose analysis kit was purchased from Young-Dong Pharmaceutical Co. Ltd. (Seoul). Cholestezyme-V, HDL-cholesterol assay kit, and triglyzyme-V (Eiken) kit were purchased from Shin-Yang Chem. Co. Ltd. Other reagents and solvents were first grade commercially available.

Animals

Male ICR mice weighing 20-30 g and male Sprague-Dawley rats weighing 200-250 g bred in the animal facility of Natural Products Research Institute were used. All animals were acclimated at least for one week, and fed

with a diet of animal chow and water *ad lib.* They were housed at 23 ± 0.5°C and 10% humidity in a 12 hr light-dark cycle.

EP-induced hyperglycemia in mice

Groups of 8 mice were administered i.p. with AS dissolved in 0.9% NaCl or vehicle alone. Four hours later, EP (0.6 mg/kg, i.p.) was administered and blood samples were withdrawn by decapitation 1 hr post dosing for blood glucose determination. Serum glucose levels were measured using glucose oxidase kits (Young Dong Pharm Co. Ltd.).

Measurement of glycogen content in mouse liver

In brief, groups of 10 mice were administered i.p. with 100 mg/kg of AS before induction of epinephrine-induced hyperglycemia. Animals were killed by decapitation and livers were removed for the measurement of glycogen contents. To the liver, 30% KOH solution (100 mg/2 ml) was added and the mixture was heated in a boiling water bath for 20 min. Ethanol was added to the mixture, which was then kept at 4°C overnight. The mixture was centrifuged at 3000× *g* for 15 min, fractionated twice with ethanol. The combined precipitate was dried in a desiccator, dissolved in water, and glucose content was measured using the anthrone-sulfuric acid method (Johnson and Fusaro, 1996).

Oral glucose tolerance test in normal mice

Groups of 10 mice were fasted overnight and administered i.p. with AS. Control mice received 0.5 ml of vehicle (0.9% NaCl) alone. Four hours later, a glucose load of 2 g/kg was given and blood samples were withdrawn by heart puncture from three or four mice of each group at 30, 60 and 120 min after glucose load. Glucose levels were measured in 20 μl of serum using glucose oxidase kits (Mariam *et al.*, 1996)

Weight-loaded forced swimming performance test

ICR mice weighting 25-30 g were administered with test samples orally for 5 consecutive days and 24 hr later, the swimming test were performed (Moriura *et al.*, 1996). Mice were divided into three groups of 9 animals each. In all experiments the swimming test was carried out with the tail weight [(b.w.+3 g)× 0.065 g] attached at 5 cm from the base of the tail of the animals. A stainless water tank, divided into 9 separate compartments with 15 cm by 13 cm by 25 cm deep, equipped with a circulation pump and a thermostatically controlled heating unit was used maintaining the temperature at 33 ± 2°C. The swimming time was defined as the interval between the onset of swimming and the point at which the animal became fully submerged for 5 sec. α-Tocopherol acetate was used as a positive control substance and it was administered orally, suspending in gum arabic (5 g/dl).

Carbon clearance test

Carbon clearance test was carried out according to the method established by Wagner *et al.* (1984) with slight modification. ICR mice weighing 20-30 g were administered i.p. with test compounds dissolved in phosphate buffered saline solution for 3 consecutive days and 24 hr after the last treatment of the samples, each mouse was injected i.v. with carbon suspension (Rotring, diluted 8 times with PBS containing 1% gelatin and warmed at 37°C) at a dose of 10 μl/b.w. 3, 6, 9 and 12 min after the injection. Then, blood was withdrawn from the orbital vein into 0.1% sodium carbonate (2 ml) and carbon concentration in blood was estimated spectrophotometrically by determining the absorbance at 660 nm. From the absorbance, the linear regression coefficient (RC) was calculated plotting –log E against time. The potencies of carbon clearance was expressed as the rate of regression coefficient of the animals treated (RCtr) to those of the control (RCc). Zymosan was used as a positive reference compound which was injected i.p. at 50 mg/kg dissolved in PBS solution.

Measurement of acid phosphatase in murine macrophages *in vitro*

The activity of acid phosphatase in murine macrophages was assayed by the procedure of Suzuki *et al.* (1990). In brief, murine peritoneal macrophages were elicited by i.p. injection of 2 ml of sterile 2.98% Brewers thioglycollate medium into the cavity of ICR mice and peritoneal exudates cells were obtained 3 days after injection by peritoneal lavage with ice-cold RPMI 1640 medium. Cells were washed twice, and resuspended in RPMI 1640 medium, supplemented with 5 mM Hepes, 10% FBS, penicillin (100 units/ml) and streptomycin (100 μl/ml). On average, 15-30 $\times 10^6$ macrophages were obtained from each mouse. The total cell number was counted using a hematocytometer, and cell viability was examined by the Trypan blue exclusion method. A 200 μl of the cell suspension, containing 2×10^5 cell/well, was placed in a 96 well culture plate. The cells were allowed to adhere for 2 hr at 37°C in a humidified 5% CO_2 incubator. Non-adherent cells were removed by washing twice with RPMI-1640 medium. Fresh media and test samples were added to each well and the plates incubated with macrophages at 37°C for 24 hr. The medium was discarded by aspiration, and the macrophage monolayer in each well was solubilized by addition of 25 μl of 0.1% Triton X-100. After addition of 200 μl of *p*-nitrophenyl phosphate/0.1 M citrate buffer (pH 5.0) to each well, the plate was incubated at 37°C for 60 min. The reaction was stopped by addition of 50 μl of 0.2 M borate buffer (pH 9.8) and the absorbance measured at 405 nm.

Measurement of lipid in the serum

The serum cholesterol and triglyceride contents were estimated according to the procedures described by Kimura *et al.* (1981). Rats were pretreated with cholesterol rich diet before the commencement of the experiments. Control rats were given corn oil (10 ml/kg, b.w.) containing 15% cholesterol and 1% sodium cholate orally for 3 consecutive days with standard lab. chows and tap water *ad. lib.* The experimental animals received AS and chitosan oligosaccharide as a positive reference drug dissolved in corn oil at a dose of 100 mg/kg/day for 7 days. Blood was taken from the ocular vein 24 hr after the last administration of the test compounds and centrifuged at 1000 rpm for 10 min to separate serum. Total cholesterol, HDL-cholesterol and triglyeride in the serum were determined using cholestezyme-V kit, HDL-cholesterol assay kit (HDL-C555, phosphotungstic acid-Mg^{2+} precipitate) and triglyzyme-V (Eiken, Shin Yang Chem. Co. Ltd.), respectively.

Statistics

Statistical comparisons between groups were performed using the Students *t*-test. Significance was accepted at $p < 0.05$.

RESULTS

Effect on EP-induced hyperglycemia and liver glycogen contents in mice

The effects of AS on EP-induced hyperglycemia in mice were tested and the results were indicated in Fig. 1. EP produced a highly significant hyperglycemic response by approximately 106% increase in the blood glucose level compared with that of the normal control, when measured 1 hr after the administration of EP (0.6 mg/kg, i.p.). A single treatment with AS (100 mg/kg, i.p.) was shown to cause a significant reduction (47.2%) in hyperglycemic effect induced by EP, which was stronger than tolbutamide (36.0 %), a positive reference drug. It can be seen that EP, on the other hand, lowered significantly the liver glycogen level of the control mice compared to the normal animals, but some significant reversal of its levels was observed by AS treatment (Fig. 2).

Effect of the glucose tolerance in mice

The glucose tolerance test in the normal mice was carried out and the results are shown in Fig. 3. With oral glucose load at 2 g/kg in the control mice, a marked elevation of glucose level was attained up to approximately 333 mg/dl and then decreased to around 180-155 mg/dl level and sustained its level up to 3 hr after the glucose load. When treated at 50 and 100 mg/kg i.p., a significant suppression of the rise in the serum glucose was observed time dependently from 30 min up to 2 hr after glucose load. At a dose of 50 mg/kg of AS, a gradual decrease in glucose level was observed and reached around normal level at 2 hr. At a dose of 100 mg/kg, almost complete suppression of glucose level (110 mg/dl) was observed even at 1 hr after the glucose load attaining normal glucose level at 2 hr.

Effect on weight-loaded forced swimming time in mice

When AS was administered orally at a dose of 100 mg/kg/day once a day for 5 consecutive days, it caused a significant prolongation of forced swimming performances by 136% compared with those of the control, which reflects antifatigue effect of AS. The mean swimming time was

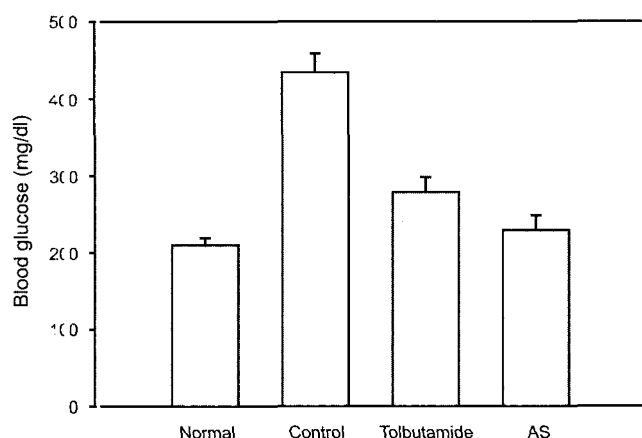

Fig. 1 Effect of AS on blood glucose level of EP-induced hyperglycemic mice. Mice were administered i.p. with AS (100 mg/kg) and tolbutamide (50 mg/kg) dissolved in saline. Four hours later, EP (0.6 mg/kg, i.p.) was administered and blood glucose level was determined one hour later. Each value represents the mean ± S.E.M. from 10 mice. Significantly different from the control; *p<0.01, **p<0.001.

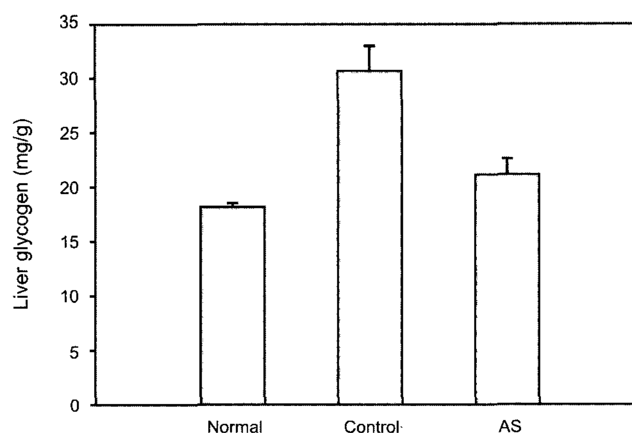

Fig. 2. Effect of AS on liver glycogen levels of EP-induced hyperglycemic mice. Mice were administered i.p. with AS (100 mg/kg) dissolved in saline. Four hours later, EP (0.6 mg/kg, i.p.) was administered and blood glucose was collected one hour later. Each value represents the mean ± S.E.M from 10 mice. Significantly different from the control; *p < 0.001.

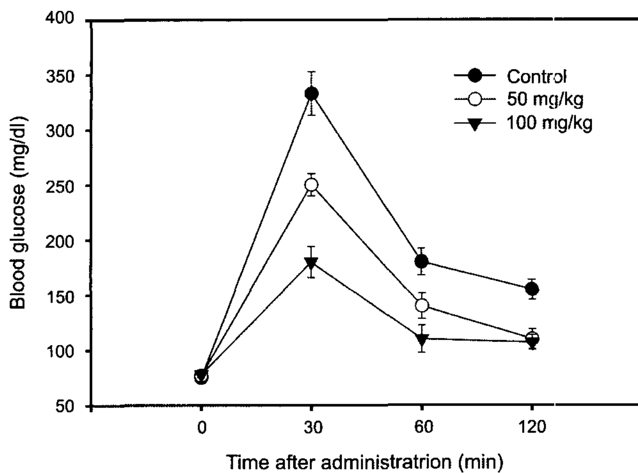

Fig. 3. Glucose tolerance test of AS in mice. Normal mice were fasted overnight (18 hr) and administered i.p. with AS. After 4 hr, glucose (2 g/kg) was given orally and serum glucose level was estimated at definite intervals. Each value represents the mean ± S.E.M. Significantly different from the control; *$p < 0.01$, *$p < 0.001$.

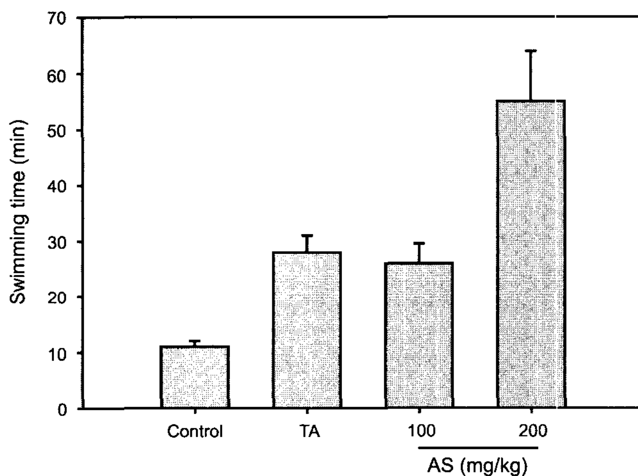

Fig. 4. Effect of AS on weight-loaded forced swimming performance in mice. Mice were administered orally with AS for 5 consecutive days and the swimming time was evaluated 24 hr after the last treatment of the test samples. Each value represents the mean ± S.E.M. from 10 mice. TA; α-tocopherol acetate (100 mg/kg), Significantly different from the control; *$p < 0.01$, **$p < 0.001$.

almost the same as that of α-tocopherol acetate, the positive reference drug. At 200 mg/kg dose level, the group treated with AS showed a remarkable prolongation of swimming time by 400% increase compared to the control.

Effect on immune functions in mice

AS exhibited a significant enhancement of the carbon clearance representing phagocytosis of macrophages *in vivo*. The potencies of phagocytosis expressed as the regression coefficient ratio (RCtr/RCc), were 1.33 and 1.38 at 50 mg/kg/day and 100 mg/kg/day, respectively. These

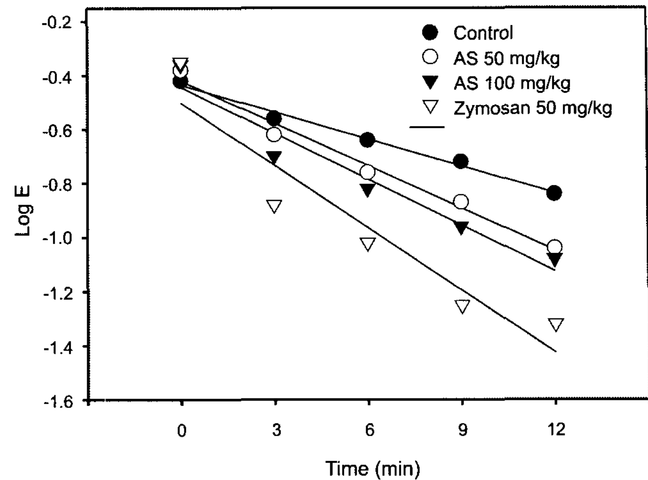

Fig. 5. Effect of AS on carbon clearance in mice. Mice were administered i.p. with test compounds for 3 consecutive days and 24 hr after the last treatment of the sample, each mouse was injected i.v. with carbon suspension (Rotring, diluted 8 times with PBS buffer containing 1% gelatin and warmed at 37°C) at dose of 10 μl/b.w. Blood was withdrawn from orbital vein into 0.1% sodium carbonate at 3 min interval up to 12 min after the injection, and the carbon concentration in blood was estimated by determining the absorbance at 660 nm. From the absorbance, the liner regression coefficient (RC) was calculated plotting log E against time. The immuno-stimulating activity was expressed as the rate of regression coefficient of the animals treated (RCtr) to those of the control (RCc). Regression coefficient ratio (RCtr/RCc): zymosan, 1.41, active; acharan sulfate, 50 mg/kg, 1.33, active; 100 mg/kg, 1.38, active.

activities were a little weaker than that of zymosan (RCtr/RCc = 1.41), known as a typical phagocytosis enhancer (Fig. 5). The effect of AS on the acid phosphatase activity, a marker enzyme representing lysosomal ezymes in macrophages, were tested *in vitro*, and the results were shown in Fig. 6. As shown in Fig. 6, AS was demonstrated to exhibit on acid phosphatase, e.g., at lower concentrations (1-20 μg/ml), it exhibited a significant enhancement of the enzyme activity both in the presence or absence of LPS compared to that of the control, but at higher concentrations (50-100 μg/ml), it caused a significant inhibition of the enzyme activity in a concentration dependant manner.

Effect on the serum lipids in rats

As shown in Fig. 7, both total cholesterol and triglyceride level in the serum were shown to be significantly elevated in the group treated with cholesterol rich diet up to 17% and 96%, respectively, as compared to those of the normal animal group. With consecutive 5 daily oral treatments at 100 mg/kg/day, however, AS was found to cause significant decrease in total cholesterol and triglyceride by 19.0 % and 25.9%, respectively as compared to those of the control animals. HDL-cholesterol, on the other hand, increased by about 100% compared to that of the control.

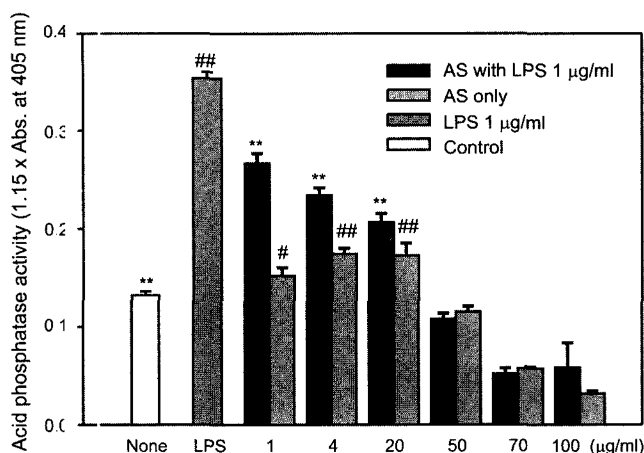

Fig. 6. Effect of AS on acid phosphatase activity of peritoneal macrophage Mouse peritoneal macrophages were seeded into 96-well culture plate at densities of 2×10^5. After 2 hr, non-adherent cells were removed and the medium was refreshed. The samples dissolved in 0.2% DMSO were incubated with macrophage monolayer at 37°C for 24 hr. The activity of acid phosphatase was calculated as follows: Acid phosphatase activity (*p*-nitrophenol µmol/2×10^5 macrophages/60 min) = $1.15\times$ absorbance at 405 nm. Statistical significance: $*p < 0.05$, $**p < 0.01$ vs. LPS control; $\#p < 0.05$, $\#\#p < 0.01$ vs. non-stimulated control.

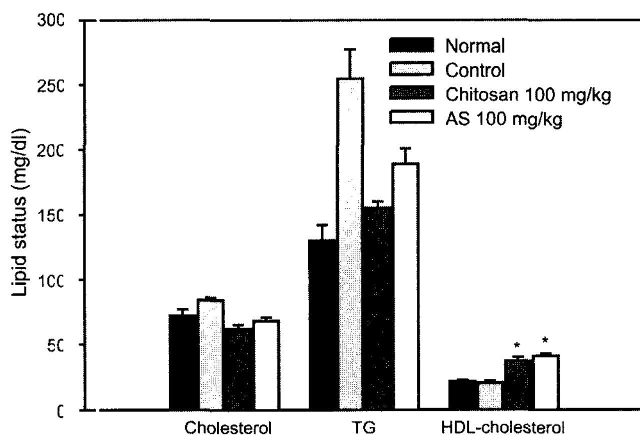

Fig. 7 Effect of AS on serum total cholesterol, triglyceride and HDL-cholesterol levels in rats. All groups were given corn oil (10 ml/kg/b.w) containing 15% cholesterol and 1% sodium cholate (cholesterol-rich mixture) orally for 10 days, then AS was treated for 5 consecutive days. Blood was taken 12 hours after the last administration of AS. Significantly different from the control; $*p < 0.01$, $**p < 0.001$.

DISCUSSION

The EP-induced hyperglycemia may be explained in terms of changes in the effects of catecholamines i.e., enhanced glycogenolytic effects of EP, which is principally responsible for the acute hyperglycemia produced by this hormone (Kreismann, 2000). The present results demonstrated that EP caused a marked elevation of blood glucose level as well as a significant reduction in the liver glycogen levels in the control animals. We can postulate that a significant decrease in the blood glucose and a significant reversal of liver glycogen contents in AS treated group in the present study occurred due to the inhibition of glycogen break down in the liver. The inhibition of glyconeogenesis, the increase in the utilization of peripheral glucose or the direct inhibition of insulin release in the liver might be another reasons for the hypoglycemic effect of AS. The fuel such as glucose, amino acids and fatty acids, where disposition is controlled by the islet hormones among which glucose is a major signal for insulin secretion and thus insulin reserve is widely assessed by the glucose tolerance test over a fixed period of time after glucose load. The sustained elevation and maintenance of blood glucose during fasting mainly depend on glucagon-stimulated gluconeogenesis as well as inhibited insulin secretion. A dose dependent lowering effect of blood glucose level in mice treated with AS might primarily indicate that a direct enhancement of insulin release by AS.

Among immune cells, macrophages play major roles in the immune system, such as in antigen-presentation, phagocytosis, cytokine production, and inflammation (Marcil *et al.*, 2002). Regulating macrophages functions might enable us to understand and control the immune system. The present results demonstrated that AS caused a significant enhancement of the phagocytosis as measured by the carbon clearance *in vivo*. This clearly indicated that a significant activation of the immune cells such as macrophages by AS, which reflects an immuno-stimulating activity of AS. The fact that AS exhibited a dual effect in the acid phosphatase activity in the present experiment, suggested that AS, at the lower concentration, acts as an immuno-suppressor. We previously observed that AS exhibited as anticancer activity as measured by the growth of the solid tumor induced by sarcoma-180 ascitic tumors in mice and the further mechanism demonstrated that the anticancer activity of AS attributed to its anti-angiogenic activity (unpublished data). Although a precise mechanism has yet elucidated, we can explain that AS possesses both immuno-stimulating and tumor inhibitory properties. A significant prolongation of the forced swimming performance by AS can also be explained that AS was able to increase in the endurance of the animals due to its immuno-stimulating activities.

It is known that a high fat diet causes a fatty liver and induces hyperlipemia in rats.

The ultimate purpose of the treatment of hyperlipemia is in the prevention of hypoxic symptoms such as angina pectoris and acute myocardial infarction by suppressing arteriosclerosis. In the presents experiments, it was demonstrated that oral administrations of an oil mixture containing high cholesterol as well as triglyceride, elevating HDL-cholesterol level. It has also been reported that the

elevation of serum HDL-cholesterol is an anti-arteriosclerotic factor. These results suggest that AS may be effective for the treatment of hyperlipemia as well as arteriosclerotic symptoms.

In conclusion, AS has various biological activities and can be therapeutically important target molecule in the body. Further research is required to study its mechanism at the molecular level.

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