

## Full Length Research Paper

## Anti-diabetic effects of fermented *Acanthopanax senticosus* extracts on rats with streptozotocin-induced type 1 diabetic mellitus

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As a consequence of increased obesity prevalence, diabetes mellitus (DM) has become one of the most common diseases in humans. *Acanthopanax senticosus* is a species of small, woody shrub in the family Araliaceae and has been used as a medicinal plant. In a preliminary study, we found that extracts from *A. senticosus* fermented with *Phellinus linteus* (ASPL) had the most potent effects against diabetes as compared to other fermented or crude extracts. In the present investigation, the effects of ASPL on mice and rats with streptozotocin (STZ)-induced diabetes was evaluated. Type I DM was induced by intra-peritoneal injection of STZ that has direct toxic effect on pancreatic  $\beta$  cells in mice and rats. Seven days after injection, blood glucose level of the diabetic mice was significantly higher than those of the control animals (387.6 and 85.5 mg/dl, respectively). ASPL was orally administered for 14 days in STZ-induced diabetic mice. Daily administration of the extracts for 14 days significantly reduced blood glucose levels of the diabetic mice (213 mg/dl), whereas glucose levels of the untreated diabetic mice were unchanged (404 mg/dl). Moreover, serum levels of alkaline phosphatase (ALT), aspartate aminotransferase (AST), total cholesterol, and low-density lipoprotein (LDL) were significantly reduced in ASPL-treated mice comparing to the diabetic control. Oral administration with ASPL also reduced weight of thrombi in the arteriovenous shunt model of rat. In conclusion, our data suggest that fermented *A. senticosus* extract can ameliorate diabetes, hyperlipidemia, and thrombogenesis.

**Key words:** Type 1 diabetes mellitus, *Acanthopanax senticosus*, fermentation, thrombosis, oxidative stress.

### INTRODUCTION

The population of people with diabetes mellitus (DM) is increasing rapidly worldwide. DM is a chronic disease characterized by hyperglycemia and dyslipidemia with disrupted carbohydrate and protein metabolism. Type 1 diabetes (insulin-dependent DM) is caused by deficient insulin secretion by  $\beta$  pancreatic cells. Type 2 DM (non-insulin-dependent DM) is characterized by insulin

resistance. The administration of insulin or synthetic hypoglycemic agents used to treat type 2 DM can produce many undesirable effects (Thakkar and Patel, 2010). Therefore, there is increasing demand for natural products with anti-diabetic effects (Park et al., 2012). It has been documented that administration of the medicinal plant exerts hypoglycemic effect while alleviating

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liver and renal damage associated with streptozotocin (STZ)-induced DM in rats (Kaleem et al., 2008).

*Acanthopanax senticosus* (Araliaceae), also known as Siberian ginseng, is a hardy shrub approximately 2-m high that is native to the far Eastern Asia and has been used in traditional oriental medicine (Huang et al., 2011). It has been found that this plant has anti-fatigue effects (Huang et al., 2011; Zhang et al., 2011) and hypoglycemic activities through the inhibition of glucose absorption (Watanabe et al., 2010). Although several studies have demonstrated the effect of *A. senticosus* on type 2 diabetes (Hong et al., 2009; Yuan et al., 2012), the effect on type 1 DM has not been extensively investigated. The present study, therefore, examined the effect of *A. senticosus* on STZ-induced type 1 DM and evaluated the accompanying changes in enzymatic activities.

## MATERIALS AND METHODS

*Phellinus linteus* (KCTC 6719) as a standard strain was acquired from the Gene Bank of the Korea Biological Resource Center (Daejeon, Korea). The mycelium was cultivated in potato dextrose agar (PDA; BD, Detroit, MI, USA) for 15 days. *A. senticosus* was purchased from Hyokwang Bio-Chem Inc. (Wonju, Korea) in 2009 and 2010.

### Cultivation conditions for manufacturing liquid spawn cultures

*P. linteus* mycelium (in all liquid cultures) were incubated in PDA medium for 7 d at 28°C. Discs (8 mm in diameter) were created by cutting with a cork borer. Five to six discs were placed in an Erlenmeyer flask containing 100 ml of potato dextrose broth (PDB; BD) and then cultured in a shaker (SI-400R; Jeiotech, Daejeon, Korea) for 6 days. The culture was then homogenized using a grinder (LB10S; Waring, Torrington, CT, USA) and 9 ml of the homogenate was subsequently transferred to an Erlenmeyer flask in which 100 ml of PDB was left for 5 days. These cultures were used as the main spawn.

### Cultivation of *P. linteus* mycelium in a natural

To obtain *A. senticosus* extract fermented by *P. linteus* (ASPL) mycelium, fresh *A. senticosus* stems and leaves were washed, peeled, and ground. The ground *A. senticosus* stems (50%) and leaves (50%) were placed in a fermenter and sterilized for 20 min at 120°C. The temperature of the fermenter was rapidly decreased to 25°C and the resulting *A. senticosus* solution was used as a natural medium. The fermented *A. senticosus* product was obtained by cultivating 9% of the *P. linteus* liquid spawn at an optimum temperature. The *A. senticosus* solution was fermented by *P. linteus* mycelium in Gashiohgraphi substrate, extracted with 70% ethanol at 85°C for 6 h, and filtered through filter paper (Adventec 5eA; Advantec, Tokyo, Japan). The filtrate was then evaporated and freeze-dried (Ilsinbiobase, Dongcheon, Republic of Korea). The freeze-dried fermented *A. senticosus* product was used to treat the diabetic rats and mice.

### Animals

Male Sprague Dawley (SD) rats (60 days old and weighing 240 to 250 g) and male ICR mice (52 days old and weighing 22 to 25 g)

were obtained from Orient Co. (Seoul, Republic of Korea). The animals were maintained in a standard laboratory animal facility with free access to feed and water. All experimental procedures and protocols used in this investigation were reviewed and approved by the Ethics Committee of Kyungpook National University (Daegu, Republic of Korea).

### Induction of type 1 DM

Type 1 DM was artificially induced by a single intraperitoneal (IP) injection of a freshly prepared STZ solution (Sigma, St. Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.5) and delivered at a concentration of 200 mg/kg body weight for the rats and 70 mg/kg body weight for the mice. The animals were fasted overnight prior to STZ administration. Control mice and rats received an IP injection of citrate buffer alone. Three days post-STZ administration, mice or rats with stabilized diabetes as indicated by fasting blood glucose levels of more than 250 mg/dl were selected for the study. Treatment was started 4 days after STZ administration and continued for 14 days.

### Treatment of diabetic mice and rats with ASPL and ASHE

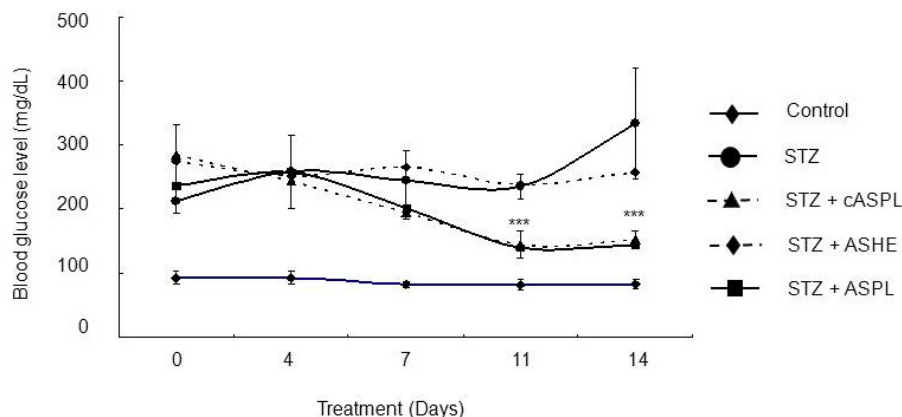
For the preliminary study, 22 mice were randomly assigned to the following five groups: (1) control, (2) diabetic (STZ), (3) diabetic treated with non-fermented *A. senticosus* extract (ASPL) with *P. linteus* (STZ + crude ASPL), (4) diabetic treated with fermented *Hericium erinaceus* extract (STZ + ASHE), and (5) diabetic treated with fermented ASPL (STZ + ASPL). Eleven rats also were divided into the three following groups: (1) control, (2) diabetic (STZ), and (3) diabetic treated with fermented ASPL (STZ + ASPL). At the end of the experimental period, the mice and rats were anesthetized and sacrificed by cervical dislocation. Blood samples were collected; the serum was isolated by centrifugation (1000 ×g for 20 min at 4°C) and subjected to biochemical analysis.

### Thrombus formation with an extracorporeal shunt

*In vivo* antithrombotic activity of ASPL was evaluated in a rat extracorporeal shunt model as previously described by Umetsu and Sanai (1978) with slight modification. Briefly, 1 h after administration of ASPL (125, 250, and 500 mg/kg, p.o.) or vehicle, non-fasted male rats were anaesthetized with urethane (1.75 g/kg, IP) and an incision was made over the trachea. The right jugular vein and left carotid artery were exposed, and the two ends of the extracorporeal shunt were inserted into them. The shunt consisted of two 12-cm sections of polyethylene tubing (with a 0.81-mm and 0.58-mm external and internal diameter, respectively) connected with 5-mm silicone rubber plugs to a 6-cm section of polyvinyl tubing (3-mm internal diameter). A 6-cm length of cotton thread was secured between the two plugs so that it was oriented longitudinally to the blood flow through the cannula. Before cannulation, the tubing was filled with a 0.9% (w/v) sodium chloride solution (saline). The shunt was left in place for 15 min after extracorporeal circulation was initiated. The flow was then stopped and the thread was removed. The formed thrombus was separated from the thread and unclotted blood, and weighed.

### Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical scavenging activity

A DPPH assay was performed to measure the hydrogen atom (or one electron) donating and antioxidant activities of ASPL due to free radical scavenging. Blois method (Blois, 1958) with slight



**Figure 1.** Preliminary screening revealed that ASPL has more potent effect than other extracts. ICR mice treated with STZ were found to be diabetic as indicated by fasting blood glucose levels of more than 250 mg/dl. Treatment with the test reagents was started 4 days after STZ administration and continued for 14 days. Each extract (50 mg/kg/day) was administered orally. The control animals were treated with vehicle (carboxymethylcellulose, CMC) instead of the extract. The graph shows fasting glucose levels of each group starting on the first day of treatment. Data are presented as the average  $\pm$  SEM. Glucose levels of the STZ + ASPL group and STZ + crude ASPL group were significantly reduced compared to that of the STZ group. Data are presented as the mean  $\pm$  SEM. \*\* $p < 0.01$  compared to the STZ group.

modifications was used for this experiment. DPPH, a stable free radical that is purple in color, was reduced into yellow diphenylpicryl hydrazine. ASPL was tested at different concentrations (6.25 to 400  $\mu$ g/ml) to assess dose-dependent effects. A fresh batch of a radical stock solution was prepared daily. Electron-donating activity (EDA, expressed as a percentage) indicated the difference in absorbance between the mixture and control solution:  $\text{EDA (\%)} = (\text{absorbance of the control} - \text{absorbance of the mixture}) / \text{absorbance of the control} \times 100$ .

### Statistical analysis

Differences were analyzed with a one-way analysis of variance followed by a post-hoc Dunnett's test (SAS Institute Inc., Cary, NC, USA). All data are presented as the mean  $\pm$  standard error of the mean (SEM). P values of 0.05 or less were considered to be statistically significant.

## RESULTS

### Effects of the *A. senticosus* extracts on STZ-induced diabetes in rats and mice

To evaluate the effects of ASPL, type 1 DM was induced in ICR mice with STZ. As shown in Figure 1, both the unfermented (crude) and fermented ASPL significantly suppressed blood glucose levels after 11 days of oral administration ( $p < 0.01$  for the STZ + ASPL group and STZ + crude ASPL group compared to the STZ group). Further biochemical analysis of serum samples showed that the unfermented ASPL had little effect on hepatic enzyme levels or lipid profiles (Table 1). The average AST level of the STZ group was  $806 \pm 428$  mg/dl on day 14

while the average AST level of the STZ + ASPL group was  $353 \pm 141$  mg/dl ( $p < 0.05$ ). In addition, triglyceride levels of the STZ + ASPL group were much lower than those of the STZ group ( $48 \pm 11$  versus  $126 \pm 24$  mg/dl, respectively;  $p < 0.05$ ). These findings demonstrated that ASPL was very effective for lowering serum AST and triglyceride levels in the ICR mice with STZ-induced type 1 DM.

The effects of ASPL on SD rats with STZ-induced type 1 DM was also accessed (Figure 2). The diabetic rats were given oral doses of ASPL for 14 days, and the fasting glucose levels were measured after treatment. The fasting serum glucose level of the control group was  $81.1 \pm 3.1$  mg/dl throughout the 14-day observation period. The serum glucose levels of the STZ group were significantly higher than that of the control group and increased slightly over time from  $371.5 \pm 79.3$  to  $404.0 \pm 15.0$  mg/dl during the 14-day observation period. The serum glucose level of the STZ+ASPL group was significantly lower than that of the STZ group on day 11 after treatment ( $p < 0.01$ ). Results of this study showed that ASPL administration effectively suppressed increases in serum glucose levels of the diabetic rats.

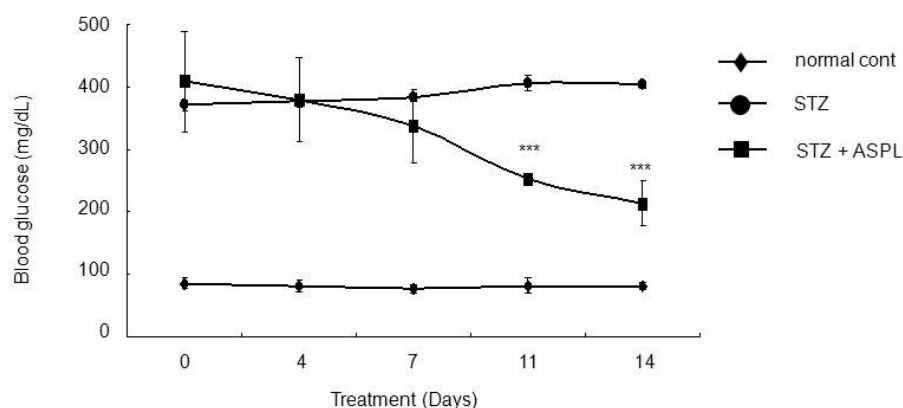
### Effects of ASPL on serum ALT, AST, total cholesterol, and low density lipoprotein (LDL) levels

To determine whether ASPL treatment affect serum ALT and AST levels or lipid profiles of the rats, a serum biochemical analysis was performed after terminating serum glucose monitoring. As shown in Figure 3, serum

**Table 1.** Biochemical analysis of serum collected from ICR mice 14 days after treatment with ASPL.

Parameter	Normal	STZ	cASPL	ASHE	ASPL
ALT (mg/dl)	21 ± 2	161 ± 69	299 ± 192	203 ± 50	106 ± 43
AST (mg/dl)	73 ± 12	250 ± 149	352 ± 162	133 ± 56	146 ± 30
ALP (mg/dl)	125 ± 11	806 ± 428	1584 ± 894	708 ± 214	353 ± 141
TG (mg/dl)	67 ± 7	126 ± 24	113 ± 3	80 ± 6**	48 ± 11**
CHOL (U/L)	110 ± 11	131 ± 13	158 ± 6	119 ± 7	93 ± 22
HDL (U/L)	27 ± 8	36 ± 1	50 ± 4	38 ± 2	30 ± 7

Type 1 DM was artificially induced by a single IP injection of a freshly prepared STZ solution. The mice were treated with the ASPL (crude and fermented) and ASHE for 2 weeks. At the end of the experimental period, the ICR mice were anesthetized and sacrificed by cervical dislocation. Blood samples were recovered and the serum was subjected to biochemical analysis. Data are presented as the mean ± SEM. Note that the crude ASPL had little effect on serum chemistry. \*\*p < 0.05 compared to the STZ group.



**Figure 2.** Daily administration of ASPL significantly decreased blood glucose concentrations in SD rats with type 1 diabetes induced by STZ (70 mg/kg) treatment, suggesting that ASPL improves hyperglycemia. SD rats with stable diabetic glucose levels (more than 250 mg/dl) were treated daily with ASPL (50 mg/kg/day) for 14 days. Each graph shows the fasting glucose levels. Data are presented as the mean ± SEM. \*\*\*p < 0.01 compared to the STZ group.

ALT and AST levels of the STZ + ASPL group were  $105.3 \pm 21.5$  and  $132.7 \pm 19.8$  U/ml, respectively. Both levels were significantly decreased as compared to those of the STZ group ( $165.3 \pm 13.0$  and  $234.0 \pm 19.8$  U/ml, respectively;  $p < 0.05$ ). The total serum cholesterol and LDL concentrations were  $93.3 \pm 9.1$  and  $11.0 \pm 2.0$  mg/ml, respectively, for the STZ group;  $49.3 \pm 9.1$  and  $3.3 \pm 2.0$  mg/ml, respectively, for the untreated control group; and  $67.0 \pm 13.1$  and  $6.7 \pm 1.9$  mg/ml, respectively, for the STZ + ASPL group. These data indicate that ASPL administration decreased total serum cholesterol and LDL concentrations although this effect was not statistically significant.

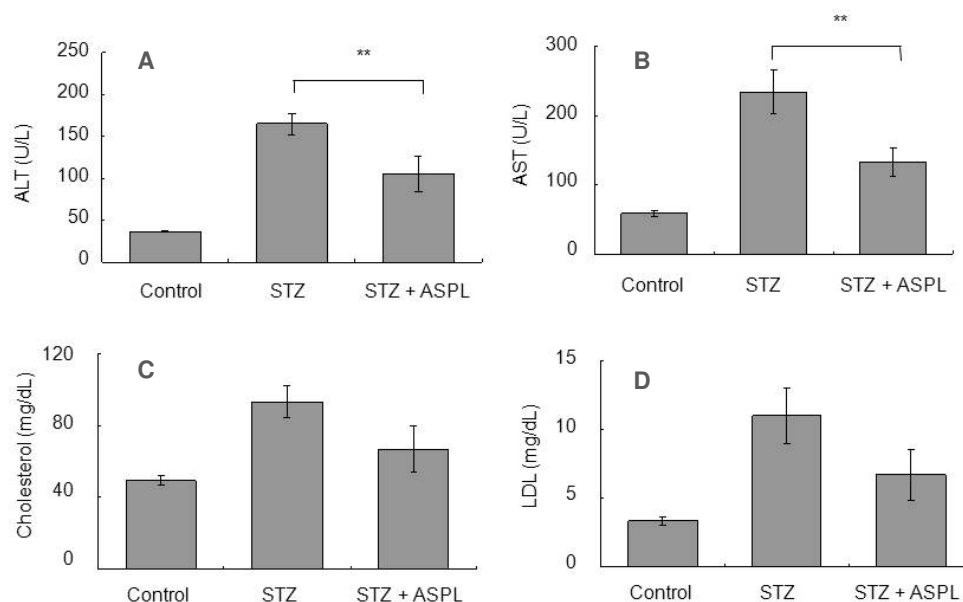
### Effects of ASPL on thrombus formation

Insulin tolerance and hyperglycemia associated with

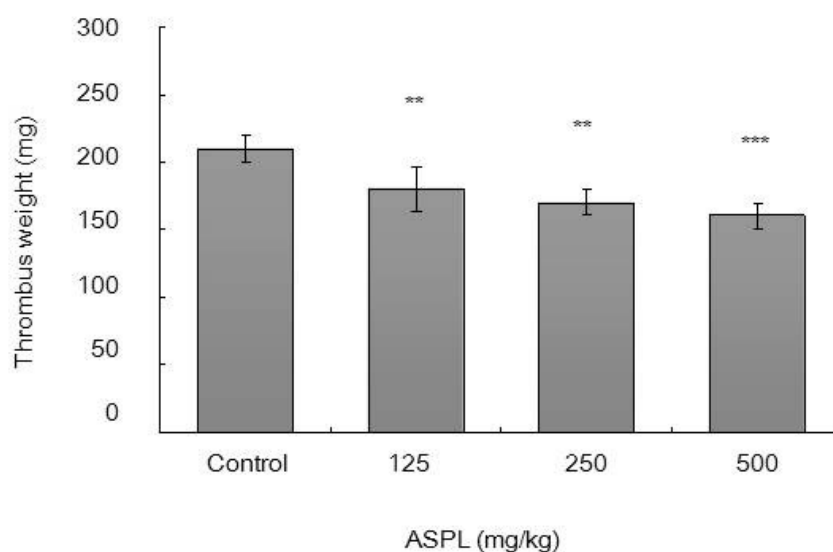
obesity or diabetes have been found to alter the levels of plasma factors such as tissue factor, intracellular adhesion molecule (ICAM-1), vascular adhesion molecule (VCAM-1), and plasminogen activator (Gerrits et al., 2010; Mathew et al., 2010; Oishi and Ohkura, 2010). It was therefore determined whether administration of ASPL affects thrombus formation. As shown in Figure 4, a single administration of ASPL decreased the weight of the thrombi formed in the rats.

### Free radical scavenging effects of ASPL

Since hyperglycemia enhances the production of reactive oxygen species (ROS) that leads to oxidative stress in endothelial cells (Lei et al., 2012; Maleki et al., 2012; Takahashi et al., 2012), the antioxidant effects of ASPL



**Figure 3.** Treatment with ASPL decreased fasting serum levels of ALT (A), AST (B), total cholesterol (C), and LDL (D). ASPL was administered orally for 14 days. At the end of the experimental period, the SD rats were anesthetized and sacrificed by cervical dislocation. Blood samples were collected and the serum was subjected to biochemical analysis. Data are presented as the mean  $\pm$  SEM. \*\* $p < 0.05$  compared to the STZ group.



**Figure 4.** Oral administration of ASPL reduced the weight of thrombi formed with an arteriovenous shunt in SD rats. The graph shows average values for at least three independent experiments. Data are presented as the mean  $\pm$  SEM. \*\* $p < 0.05$  and \*\*\* $p < 0.01$  compared to the control.

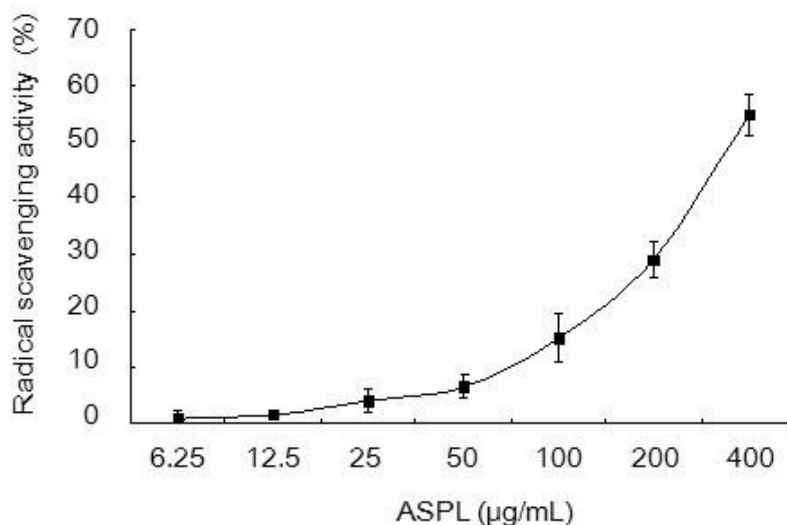
was examined. For this, the free radical scavenging activity of ASPL by monitoring DPPH radicals was measured. As shown in Figure 5, ASPL exerted free radical scavenging effects in a concentration-dependent manner. This result suggests that the antioxidant activity of ASPL may play an important role in the hypoglycemic

and anti-thrombotic effects of the extract.

## DISCUSSION

The present study demonstrated that ASPL has hypogly-





**Figure 5.** DPPH assay results showing that ASPL scavenges radicals in a concentration-dependent manner. Acetate buffer (10 mM, pH 5.5) and the fermented extracts were combined, and assay was carried out as described in 'Materials and Methods'. Each value represents the mean  $\pm$  SEM of three experiments performed in triplicate.

cemic, anti-thrombotic, and antioxidant effects in rats and mice with STZ-induced diabetes. Serum lipid and lipoprotein levels were markedly increased in both the diabetic mice and rats. Elevation of cholesterol and LDL levels is a major factor that promotes the pathogenesis of diabetes-related complications in which cells are damaged by circulating free fatty acids (Kim et al., 2010; Patel and Goyal, 2011). ASPL effectively reduced total cholesterol and LDL serum concentrations in the STZ-treated rats.

In cases of diabetes, ALT levels are elevated by hepatic cellular damage, and increase of ALT is accompanied by AST (Farswan et al., 2009). The ALT and AST concentrations indicate the health of the liver, which is an insulin-sensitive organ. Choi et al. (2006) described the ability of *A. senticosus* to protect against hepatotoxicity. Pretreatment with a glycoprotein isolated from *A. senticosus* was found to decrease ALT and ASP levels, and enhance alcohol dehydrogenase and acetaldehyde dehydrogenase activities in alcohol-treated mice (Choi et al., 2006). Similarly, in the present study, it was determined that ASPL helped prevent hepatic injury induced by hyperglycemia in the diabetic animals.

It has also been reported that elevated serum lipid levels cause cardiovascular defects (Oishi and Ohkura, 2010). However, pathology comprised from diabetes is caused by endothelial cell damage under hyperglycemic conditions. Increased levels of serum soluble adhesive molecules (ICAM and VCAM) accompanies endothelial dysfunction in patients with antiphospholipid syndrome characterized by the formation of blood vessel thrombi (Stalc et al., 2006). Therefore, it is speculated that anti-diabetic drugs modulate blood flow in diabetic animals.

The effect of ASPL on thrombogenicity was evaluated by measuring the weight of thrombi formed with an extracorporeal shunt in rats. Treatment with ASPL decreased thrombus weight in a dose-dependent manner. Our finding suggests that ASPL may ameliorate cardiovascular complications associated with diabetes.

Increasing evidence indicates that hyperglycemia accompanying diabetes increases superoxide production, thus leading to endothelial cell dysfunction (Guzik et al., 2002). Our DPPH analysis revealed that ASPL has high levels of free radical scavenging activity. ROS induces oxidative stress in cells and tissues that results in impaired function. The superoxide can directly prevent vasodilation by quenching nitric oxide in endothelial cells (Rungseesantivanon et al., 2010). Furthermore, high degrees of glucose-mediated oxidative stress interfere with glucose transport by inhibiting glucose transporters (Kim et al., 2007). Our results suggest that the antioxidant action of ASPL may play a crucial role in mediating anti-diabetic effects of the extract.

In conclusion, the present study demonstrated that ASPL helps ameliorate hyperglycemia, hyperlipidemia, impaired serum hepatic enzyme, and thrombosis formation in diabetic animals. Our key findings indicate that these anti-diabetic effects are mediated by the radical scavenging activity of ASPL. Therefore, ASPL administration may be useful for treating type 1 DM.

## ACKNOWLEDGEMENT

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and Fisheries, Republic of Korea.

## ABBREVIATIONS

**ALT**, Alanine aminotransferase; **AST**, aspartate aminotransferase; **LDL**, low-density lipoprotein; **DM**, diabetes mellitus; **STZ**, streptozotocin.

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