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RESEARCH ARTICLE

Antithrombotic Potential of Extracts from Abalone, *Haliotis Discus Hannai* Ino: *In vitro* and Animal Studies

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Abstract To screen the more promising antithrombotic abalone extracts, the *in vitro* antithrombotic activity of 6 abalone (*Haliotis Discus Hannai* Ino) extracts was examined in this study. Results of *in vitro* studies indicated that water extract of abalone viscera (WEV) presented significant longer prothrombin time (PT), partial thromboplasmatin time (APTT), and thrombin time (TT) than other abalone extracts. In addition, the platelet aggregation process was inhibited by WEV as well. The antithrombotic potential of WEV was further investigated in animal studies which revealed that WEV prolonged tail bleeding time, APTT but not PT and TT and showed no effect on the platelet aggregation and platelet number. The WEV treated rats presented lower cholesterol and triglyceride level than the control group although the difference was not statistically significant. Results of this study indicate the antithrombotic activity of abalone extracts, especially WEV, and may contribute to the further study in this field.

Keywords: antithrombotic, abalone, prothrombin time, partial thromboplasmatin time, thrombin time

Introduction

Thrombosis is a severe health problem which is the leading cause of heart attack, stroke, and many other dangerous diseases (1). The main risk factors for thrombosis are reported to be abnormal high blood lipids, hypertension, high blood glucose, cancer, elevated plasma fibrinogen, *etc* (2). Over the past several decades, tremendous attention has been paid to prevent and treat thrombosis. And several drugs such as heparin and warfarin are usually prescribed for the anti-thrombotic therapy. However, both of them have some well-known limitations. Heparin is mainly administrated intravenous and need laboratory monitoring and can occasionally cause life-threatening bleeding (3,4). As the most commonly used vitamin K antagonists, warfarin is hampered by several limitations include frequent monitoring and dose adjustment, serious bleeding, increased risk of atherosclerosis and osteoporosis (5). Therefore, functional food with anti-thrombotic activity which can be applied as supplement of the antithrombotic therapy is gaining more and more attention (6,7).

Pacific abalone, *Haliotis Discus Hannai* Ino, is an important aquatic economic species widely cultured in East Asia. For thousands of years, Korean and many other Asian peoples have used abalone as a traditional functional food. Numerous researches have been published centered on the nutritional and pharmacy values of abalone recently. González *et al.* (8) examined the nutritional value of abalone and the effects on serum cholesterol concentration in rats. Peng *et al.* (9) investigated the learning and memory improving ability of abalone extracts on mice. Bing *et al.* (10) studied the anti-tumor effect of abalone polysaccharide. Li *et al.* (11) purified a glycosaminoglycan-like polysaccharide from abalone whose anticoagulant activity was investigated *in vitro*. However, to our best knowledge, no systematic studies have been published on the antithrombotic activity of abalone.

In this study, the antithrombotic potential of 6 abalone extracts was investigated *in vitro*. Water extract of abalone viscera (WEV) which showed the best result and subsequently tested its antithrombotic ability among 6 extracts in animal studies.

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Materials and Methods

Material and chemicals Fresh abalone (*Haliotis Discus Hannai* Ino) was bought from local aquatic market in Mokpo, Korea, which was harvested in the local mariculture farm in February 2011. The abalone was shucked and eviscerated. All the viscera and muscle was gathered separately, homogenized, and stored at −20°C until use. Heparin sodium salt from bovine intestinal mucosa (≥140 USP units/mg), human thrombin (100 NIH units/mL), human fibrinogen, human plasma, and proteinase A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thrombinplastin-D and APTT-XL were obtained from Fisher Diagnostics (Middetown, VA, USA). Hypodermic syringe and blood collection tube were from Greiner Bio-One (Kremsmünster, Austria). All other chemicals were of reagent grade quality.

Preparation of abalone extracts Six abalone extracts include water extract of viscera (WEV), ethanol extract of viscera (EEV), enzymatic extract of viscera (EMEV), water extract of muscle (WEM), ethanol extract of muscle (EEM), and enzymatic extract of muscle (EMEM) were prepared as follows. WEV and WEM were carried out with 10 times (w/w) of distilled water at 37°C for 12 h, whereas EEV and EEM were processed with 10 times (v/w) of ethanol under room temperature. Proteinase A was employed in EMEV and EMEM extraction to decompose viscera and muscle at pH 7.5 and 37°C for 12 h and the fluid was boiled for 10 min in order to inactivate the protease. After extraction, all groups were filtrated and concentrated under reduced pressure. Frozen at −70°C for 48 h, all the concentrated extracts were transferred to a freeze dryer (IlShin BioBase, Yangju, Korea) and dried under vacuum over 72 h.

Coagulant parameter tests *in vitro* Coagulation parameters including prothrombin time (PT), partial thromboplasmatin time (APTT), and thrombin time (TT) were performed on a bioMerieux Coag-A-Mate MAX analyzer (Biomerieux, Inc., Jamestown, ND, USA), in accordance with the manufacturer's instructions. In brief, 100 μL of extracts at different concentrations were mixed with 100 μL of human plasma and warmed at 37°C for 180 s. After warming, 200 μL of thromboplastin-D reagent was loaded and PT was measured automatically. For APTT, the mixture of 100 μL extract and 100 μL of human plasma were warmed at 37°C for 60 s before 100 μL of APTT reagent (ellagic acid) was loaded to the test tube. It was activated for 300 s and APTT was measured after the addition of 100 μL of 25 mM $CaCl_2$. TT was determined as follows: 100 of μL extract and 100 μL of human plasma were incubated at 37°C for 60 s and it was mixed with 100 μL of TT reagent (human thrombin), then the result was recorded automatically.

Collagen induced platelet aggregation *in vitro* Platelet aggregation assay was performed according to the method of Gadi *et al*. (12). Venous blood was obtained from 1 healthy volunteer who denied taking non-steroidal or anti-inflammatory drug in the previous 2 weeks and blood sample was mixed with 3.8% trisodium citrate (9:1, v/v) in a plastic tube. Platelet rich plasma (PRP) was obtained by centrifugation of blood at 110×*g* for 10 min. Platelet poor plasma (PPP) which was used as a reference solution in aggregation assays was prepared by further centrifugation at 1,000×*g* for 20 min. Platelet aggregation was conducted at 37°C and 1,200 rpm stirred by an magnetic bar on an aggregometer, Chrono-Log 700-4 (Chrono-Log Corp., Havertown, PA, USA). In brief, 450 μL of PRP was warmed with 50 μL of abalone extract for 1 min. After that, 1 μL of collagen (1 mg/mL) which was used as platelet aggregating agent was added to start the reaction. Platelet aggregation was recorded and calculated automatically at 6 min after platelet stimulation.

Animal studies
***Animal treatment*:** Male Sprague-Dawley (SD) male rats were purchased from Deamul Science Inc. (Daejeon, Korea). They were 5-week old and all weighed 200±15 g upon purchase. The rats were housed at constant humidity (55±5%), room temperate (25±1°C), and 12 h light/dark cycles. Water and meals were provided *ad libitum*. After 7-day acclimatization, the rats were divided into 3 groups randomly for the following 5 weeks study. They were: (i) control group, orally administrated with distilled water (0.5% of BW), (ii) high concentration group, orally administrated with 400 mg/kg BW of the selected abalone extract solution, and (iii) low concentration group, orally administrated with 200 mg/kg BW of the selected abalone extract solution. The rats were fasted for 12 h before the experiments.
***Tail bleeding time assay*:** Tail bleeding time was carried out in accordance with the method described before (13). After the rats were fasted for 12 h, a cut was made using a scalpel at 2 mm from the tip of the rat tail (with a tail diameter of approximately 1 mm). The bleeding time was measured when the tail cut was done and during the test blood was blotted from the incision with a tissue paper at intervals of 30 s. Tail bleeding time was recorded until there was no obvious blotting on the tissue paper.
***Blood clotting time assay*:** The blood clotting time assay was carried out as previously described (14) with some modifications. Three drips of blood were dripped on a clean petri dish and at the same time a stopwatch was started. The blood drip was gently teased from the edge to the center with a clean $9^{\#}$ needle in order to check for blood

clot every 20 s. When the fibrin filament was observed on the tip of the needle, the stopwatch was stopped and the blood clotting time was recorded.

Coagulation parameter tests in vitro: Venous blood samples were drawn at the caudal vein and the blood was transferred to a tube containing 3.8% trisodium citrate solution used as anticoagulant. Plasma was collected after centrifugation at 1,000×g for 15 min. PT, APTT, and TT were determined using the same method described above.

Platelet aggregation assay and platelet count in vitro: Platelet aggregation test was done according to the measurement described above. Platelet count was analyzed in Saint John's Hospital in Gwangju using differential Wright's stained peripheral blood smears and phase contrast light microscopy techniques (World Precision Instruments Inc., Saratosa, FL, USA).

Triglyceride and cholesterol level analysis: There are profound evidences that cholesterol, triglyceride, HDL and LDL level correlate with blood coagulation cascade (15,16) so they were analyzed in this study. Total serum cholesterol, triglyceride, and HDL were tested in the same hospital using the standard analytical techniques. LDL concentration was calculated by Friedewald equation (17):

$$\text{LDL}=\text{Total serum cholesterol}-\text{HDL}-\text{triglyceride}\times\frac{1}{15}$$

Statistical analysis To verify the statistical significance of the studied parameters, data were expressed as means and standard deviation (mean±SD). Comparisons were made using the one-way analysis of variance (ANOVA). The p-values of <0.05 was considered a statistically significant difference.

Results and Discussion

Coagulant parameter tests *in vitro* Few studies have been published about the effects of abalone extracts on the coagulation process. Our study initially examined the effect of the 6 abalone extracts on coagulant parameters *in vitro*. The abalone extracts showed longer PT, APTT, and TT than the control group in dose-dependent manner (Table 1-3). In the PT assay, viscera extracts increased the clotting time longer than the muscle extracts on the whole. At the concentration of 0.5 and 1%, EMEV increased PT much longer than other extracts. But at the concentration of 2%, the difference between WEV and EMEV was eliminated which prolonged PT to 22.80±0.20 and 23.85±1.05 s, respectively. Furthermore, in the APTT assay, at the concentration of 2% WEV showed significant longer clotting time (115.40±3.70 s) than the EMEV (59.35±0.95 s) and the other 4 extracts. In the TT test, WEV presented significant longer TT than other extracts at the concentration of 0.5 and 2%. Although at the concentration of 1%, the TT of WEV was lower than that of EMEV, the difference was not significant. The results led us to believe WEV as a better anti-thrombotic extract than the other extracts.

A recent study isolated a glycosaminoglycan-like polysaccharide from abalone viscera which was proved to prolong APTT and PT but not TT *in vitro* (10). In our study we have demonstrated the anti-coagulation potential of abalone extracts. *In vitro* tests, WEV presented much longer clotting time than other extracts. Unlike the study mentioned above, we have proved WEV can prolong not only APTT and PT but also TT.

Table 1. Prothrombin time of abalone extract and heparin solutions (s)

Test group[1)]	Concentration			
	0.5%	1.0%	2.0%	
WEM	15.70±0.10[c2)]	15.60±0.20[cd]	16.80±0.00[b]*	
EEM	15.10±0.10[d]	15.00±0.40[d]	16.00±0.20[b]	
EMEM	15.80±0.20[c]	14.85±0.75[d]	16.10±0.10[b]	
WEV	16.90±0.55[b]	18.30±0.20[b]	22.80±0.20[a]*	
EEV	16.05±0.15[c]	16.45±0.15[c]	17.80±0.10[b]*	
EMEV	18.10±0.10[a]	20.45±0.25[a]	23.85±1.05[a]*	
Control	15.00±0.10			
Heparin	0.83[3)]	1.24	1.65	1.98
	20.75±0.75	23.30±0.01	28.55±0.15	41.75±0.55

[1)]WEM, water extract of muscle; EEM, ethanol extract of muscle; EMEM, enzymatic extract of muscle; WEV, water extract of viscera; EEV, ethanol extract of viscera; EMEV, enzymatic extract of viscera
[2)]Mean±SD (n=3) in the same column with different superscripts and in the same row with * are significantly different at p<0.05.
[3)]Concentrations of heparin solutions which worked as reference were expressed as USP unit/mL.

Table 2. Partial thromboplasmatin time of abalone extract and heparin solutions (s)

Test group[1)]	Concentration		
	0.5%	1.0%	2.0%
WEM	38.05±0.25[a2)]	40.00±0.10[c]	47.15±0.25[c]*
EEM	34.05±1.25[cd]	32.95±0.65[d]	34.30±0.10[d]
EMEM	32.25±0.15[d]	30.70±0.90[d]	33.60±0.70[d]
WEV	34.70±0.20[c]	65.50±1.80[a]*	115.40±3.70[a]**
EEV	35.95±0.95[bc]	38.25±0.45[c]	22.10±0.60[e]
EMEV	39.05±0.35[a]	47.65±0.95[b]*	59.35±0.95[b]**
Control	39.55±0.05		
Heparin	0.83[3)]	1.24	1.65
	87.70±0.70	128.60±1.10	157.60±1.50

[1)]WEM, water extract of muscle; EEM, ethanol extract of muscle; EMEM, enzymatic extract of muscle; WEV, water extract of viscera; EEV, ethanol extract of viscera; EMEV, enzymatic extract of viscera
[2)]Mean±SD (n=3) in the same column with different superscripts and in the same row with * are significantly different at p<0.05.
[3)]Concentrations of heparin solutions which worked as reference were expressed as USP unit/mL.

Table 3. Thrombin time of abalone extract and heparin solutions (s)

Test group[1)]	Concentration			
	0.5%	1.0%	2.0%	
WEM	13.75±0.35[d2)]	19.45±0.75[b]*	27.50±1.00[c]**	
EEM	15.35±0.35[c]	20.40±0.70[b]*	23.65±0.35[c]**	
EMEM	8.05±0.05[e]	10.75±0.45[c]*	17.25±0.05[d]**	
WEV	22.00±0.00[a]	29.80±0.60[a]*	52.10±0.10[a]**	
EEV	9.30±0.40[e]	10.65±0.45[c]	13.55±0.05[e]**	
EMEV	19.15±0.75[b]	31.50±0.30[a]*	41.95±1.45[b]**	
Control	8.20±0.10			
Heparin	0.10[3)]	0.20	0.40	0.60
	9.65±0.35	15.65±0.05	30.00±1.10	58.15±1.70

[1)]WEM, water extract of muscle; EEM, ethanol extract of muscle; EMEM, enzymatic extract of muscle; WEV, water extract of viscera; EEV, ethanol extract of viscera; EMEV, enzymatic extract of viscera
[2)]Mean±SD (n=3) in the same column with different superscripts and in the same row with * are significantly different at $p<0.05$.
[3)]Concentrations of heparin solutions which worked as reference were expressed as USP unit/mL.

Collagen induced platelet aggregation *in vitro* Inhibiting platelet aggregation is important in prevention and treatment of thrombus since platelet plays a vital role in the development of thrombosis (18). In our study we measured the effects of abalone extracts on the platelet aggregation process in the collagen induced platelet aggregation assay. The result listed in Table 4 was determined by maximal aggregation (MA) calculated by the Agrrolink 8 program. Among the 6 extracts, WEV showed lower maximal aggregation than other extracts and WEV inhibit platelet aggregation in a dose-dependent manner. But when compared with other published results (7,12), the result of platelet aggregation can only be regarded as mediocre.

The *in vitro* results led us to believe that among the 6 abalone extracts WEV is a more promising candidate for antithrombotic activities. So we continued to evaluate the antithrombotic ability of WEV in animal study.

Animal studies To our knowledge, this study is the first one that investigated the antithrombotic potential of abalone viscera extracts using animal model. In the animal study, body weight, body weight gain, feed and water gain were measured and no significant different were observed. Moreover, the coefficients of liver, kidneys, spleen and epididymal fat pad to body weight were measured and showed no significant different across groups (data not shown). So we believe WEV administrated to rat at the dose of 400 and 200 mg/kg BW have no obvious harmful effects on SD rats in our experimental conditions. The APTT result revealed to us that rats treated with 400 mg/kg BW presented significant longer clotting time than rats treated with 200 mg/kg BW and the control group (Fig. 3). However, WEV showed no increase in PT and TT (neither at high nor low concentration) compared to the control group. But both the tail bleeding time and blood clotting time were prolonged in WEV treated groups in a dose-dependent manner (Fig. 1, 2). And WEV decreased total cholesterol and triglyceride level especially the LDL level compared to the control group although it is not statistically significant (Fig. 4). However, neither platelet aggregation nor platelet count showed difference across groups (Table 5). So the prolonged tail bleeding time and blood clotting time were not likely caused by the inhibited platelet activity or the lowered platelet number.

In the blood coagulation tests, APTT is employed to evaluate the efficiency of the intrinsic coagulation pathway. A prolonged APTT result is generally recognized as deficiency or abnormality of factor VIII, IX, XI, XII, X, and II, prekallikrein, high molecular weight kininogen (HMWK), and fibrinogen (19). On the other hand, PT is employed to evaluate the process of extrinsic pathway and is sensitive to coagulation factor V, VII, and X (20). And TT is a test of fibrin formation, which is the common pathway of blood coagulation and an abnormal TT suggest the disturbance of the last steps of coagulation (21). In the animal studies, we found WEV can potently increased APTT but presented no effect on PT and TT.

Tail bleeding time is generally recognized as a simple but useful test for platelet aggregation *in vivo*. However, several studies in the literature indicated that the relationship was not unanimous. One study showed that *Lycopus lucidus* fractions could inhibit platelet aggregation *in vitro* and *in vivo* without prolonging the tail bleeding time (22).

Table 4. Platelet aggregation results of 6 groups of abalone extracts and saline water as blank (%)

Group[1)]	WEM	EEM	EMEM	WEV	EEV	EMEV	Saline water
MA-1[2)]	88.67±0.88[a3)]	74.50±1.26[d]	85.83±0.95[ab]	69.33±0.88[e]	78.67±1.12[c]	75.83±1.54[cd]	84.83±0.95[b]
MA-2	83.17±0.87[a]	72.17±0.60[c]	75.83±1.14[b]	63.67±0.99[d]	77.84±0.95[b]	75.50±1.38[b]	84.83±0.95[a]
MA-3	77.83±0.70[b]	70.83±1.25[c]	77.17±1.19[b]	59.33±1.20[d]	75.33±1.52[b]	71.67±0.88[c]	84.83±0.95[a]

[1)]WEM, water extract of muscle; EEM, ethanol extract of muscle; EMEM, enzymatic extract of muscle; WEV, water extract of viscera; EEV, ethanol extract of viscera; EMEV, enzymatic extract of viscera
[2)]Platelet aggregation was expressed as maximal aggregation (MA): 1, low concentration (5 mg/mL); 2, medium concentration (10 mg/mL); 3, high concentration (20 mg/mL)
[3)]Mean±SD (n=5) in the same row with different superscripts are significantly different at $p<0.05$.

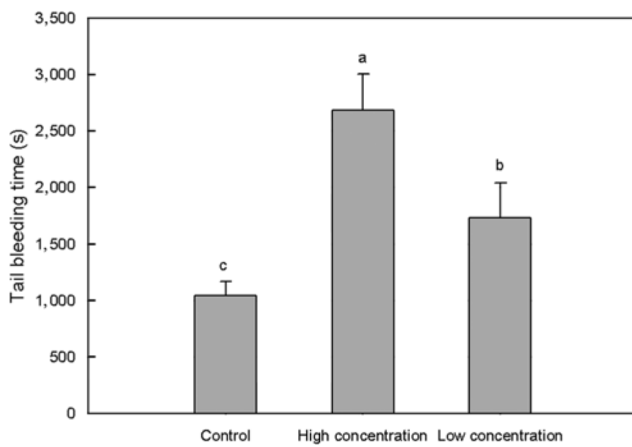

Fig. 1. Tail bleeding time of rats gavaged with distilled water (control), high (400 mg/kg BW), and low (200 mg/kg BW) concentration of WEV. Groups with different superscripts are significantly different (p<0.05).

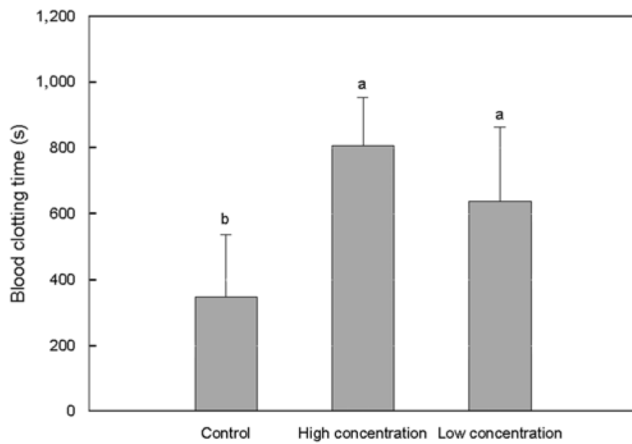

Fig. 2. Blood clotting time of rats gavaged with distilled water (control), high (400 mg/kg BW), and low (200 mg/kg BW) concentration of WEV. Groups with different superscripts are significantly different (p<0.05)

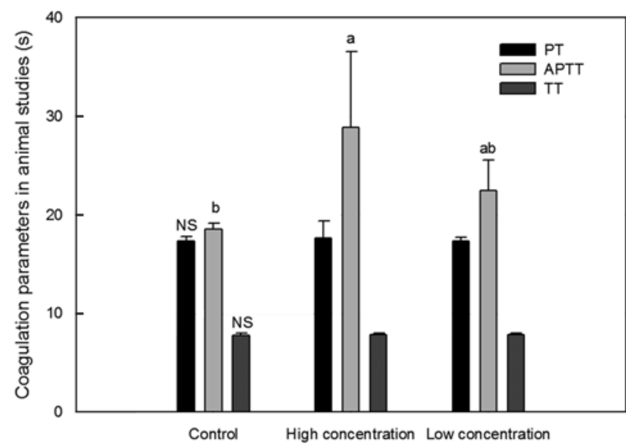

Fig. 3. PT, APTT and TT of rats gavaged with distilled water (control), high (400 mg/kg BW), and low (200 mg/kg BW) concentration of WEV. Groups with different superscripts are significantly different (p<0.05)

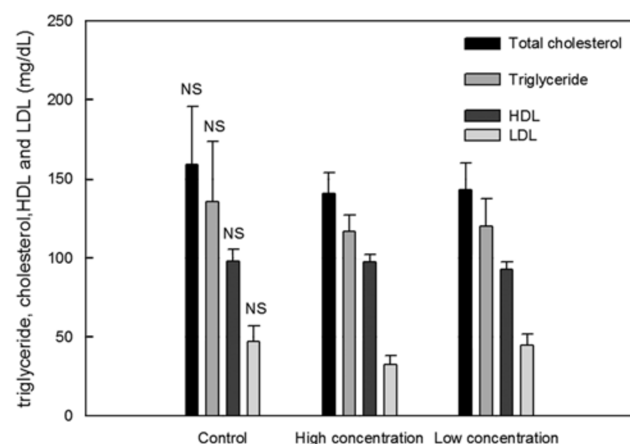

Fig. 4. Total cholesterol, triglyceride, HDL and LDL level of rat treated with distilled water (control), high (400 mg/kg BW WEV) and low (200 mg/kg BW WEV) concentration group.

Table 5. Platelet aggregation and platelet count results in the animal study

Group[1)]	Aggregation[2)]	Count (×1,000)
Control	83.60±1.21[NS3)]	1,071.00±45.94[NS]
High concentration	84.83±0.98	1,068.00±101.12
Low concentration	84.33±1.69	1,143.33±92.79

[1)]Control, orally administrated with distilled water (0.5% of BW); high concentration, orally administrated with 400 mg/kg BW of water extract of viscera solution; low concentration, orally administrated with 200 mg/kg BW of the water extract of viscera solution

[2)]Platelet aggregation was expressed as maximal aggregation (%).

[3)]Mean±SD; [NS]Not significantly different in the same row at p<0.05 (n=5)

And another research proved that quinones isolated from *Auxemma oncocalyx* Taub, while inhibited the platelet aggregation process, shortened the tail bleeding time significantly (23). Moreover, a thromboxane synthetase inhibitor is reported to prolong the tail bleeding time but have little effect on platelet aggregation (24). Since platelet aggregation was induced by collagen rather than ADP or thrombin in this study, we suppose the prolonged tail bleeding time was due to platelet aggregation induced by ADP or thrombin but not collagen. But this hypothesis may need further investigation.

Many published studies which focused on the antioxidant, anticoagulant, and immunomodulating activities of abalone extracts paid much attention to the polysaccharide (10,11, 25,26). Furthermore, many polysaccharides isolated from brown seaweed, which is the main forage of abalone, showed antithrombotic or anti-platelet activity. Chandía and Matsuhiro (27) purified a fucoidan from *Lessonia vadosa* and proved the anticoagulation activity. And Ronghua *et al.* (28) proved the anticoagulation activities of an alginate sulfate *in vitro*. In addition, several enzymes that hydrolyze polysaccharides have been isolated from

abalone viscera (29-31). So it seems that the antithrombotic activity of WEV is related to the polysaccharides from abalone viscera, but this need further work to prove.

In conclusion, we have examined the antithrombotic potential of 6 abalone extracts *in vitro*. The results indicated that WEV can potently prolong APTT, PT, and TT, and inhibited the platelet aggregation process *in vitro*. So we selected WEV as a promising candidate in the animal study. In the animal study, APTT, tailing bleeding time and blood clotting time were increased in the WEV treated rats but PT, TT, and platelet aggregation and platelet count were not affected. WEV also showed beneficial effect on the cholesterol profile, decreased total cholesterol and triglyceride level, even though the reduction was not statistically significant in the SD rat model. Although further studies are needed to elucidate on the unknown aspects, our study will offer useful information on the antithrombotic potential of abalone viscera.

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