Natural Product Sciences
5(1) : 54~59 (1999)

Immuno-stimulating, Anti-stress and Anti-thrombotic Effects of Unossified Velvet Antlers

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Abstract – Both ethanol and water extracts of unossified velvet antler were found to exhibit a significant immuno-stimulating activity as measured by carbon clearance test in mice, a remarkable anti-fatigue effect in weight-loaded forced swimming performance in mice, a significant anti-stress effect on immobilization in rats. The antler extracts also showed a weak but significant anti-thrombotic activity. These findings are indicative of adaptogenic properties of antlers and their normalizing effects during stressful condition.

Key words – Unossified velvet antler, immuno-stimmulating activity, anti-fatigue activity, anti-stress activity, anti-thrombotic activity, carbon clearance test, weight-loaded forced swimming performance, immobilization stress, platelet aggregation, anti-thrombotic.

Introduction

The unossified velvet antler of *Cervus* spp. (Cervidae in Ariodactyla) has been widely used as a valuable drug in Chinese traditional medicine from ancient times and mainly prescribed for the treatment of anemia, exhaustion, loss of vigour, cardiac and vascular system insufficiency, wounds, etc. (Hur, 1981; Pavlenko, 1969).

Although there are many reports on diversified pharmacological activities of extracts from unossified antler of different *Cervus* spp., no reliable results which demonstrate them to improve the general physiological tonus of the ailing organisms and to manifest the functional activities of the neuro-muscular as well as endocrine systems are yet obtained. The purpose of this study is to demonstrate fundamental mode of actions relating to above mentioned effects of antlers and as a first step, we report test results on immuno-potentiating, antifatigue, anti-stress and anti-thrombotic effects of water and ethanol extracts of antler.

Materials and Methods

Materials – Fresh unossified antler used in the experiments were supplied by Game Industry Board, New Zealand and were stored at -80°C until used. Zymosan, ascorbic acid, Wright's stain were purchased from Sigma Chem. Co., U.S.A. Cholesterol standard and α-tocopherol were purchased from Zunsei Chem. Co., Japan. Human blood was supplied by blood bank of Red Cross, Korea. Human platelet concentrate was purchased from Seoul National University Hospital just before each experiment. Collagen was obtained from Chrono-Log Corp., U.S.A. Platelets were counted on PLT-4 platelet analyzer, Texas International Lab. and platelet aggregation experiments were performed with a Whole Blood Lumi-aggregation System, Chrono-Log. Other reagents were of first grade commercially available.

Animals – Male Sprague-Dawley rats weighing 150-250 g and ICR mice weighing 20-30 g bred in the laboratory animal care facility of Natural Products Research Institute were used. Animals were maintained in an air-conditioned room with 12 hr day/night cycle. Lab. chows and tap water were given *ad. lib.*

Preparation of test extracts – Unossified velvet antlers were coarsely powdered and extracted refluxing on a water bath for 5 hrs with 95% ethanol 4 times and filtered. The combined ethanol solution was evaporated to dryness under reduced pressure to obtain ethanol extract (Yield = 3.2 w/w%). For preparation of water extract, the powdered antler was heated with distilled water for 3 hrs on a mantle

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heater 6 times. A clear supernatant obtained by repeated filtration and centrifugation was freeze-dried and used for experiment (Yield = 1.6 w/w%).

Carbon clearance test – The carbon clearance test was carried out according to the method described by Wagner *et al.* (1985) with a slight modification. ICR mice weighing 25-30 g were administered orally with test extracts dissolved in phosphate buffered saline solution for 4 consecutive days and 48 hr after the last treatment of the samples, each mouse was injected i.v. with carbon suspension (Rotring, diluted 8 times with Phosphate buffered saline (PBS) containing 1% gelatin and warmed at 37°C) at a dose of 10 μl/b.w. Blood was withdrawn from the orbital vein into 0.1% sodium carbonate (2 ml) 5, 10, 15 and 20 min after the injection and carbon concentration in blood was estimated spectrophotometrically by determining optical density at 660 nm. From optical density, the linear regression coefficient (RC) was calculated plotting log E against time. The immunostimulating activity was expressed as the rate of regression coefficient of the animals treated(RCtr) to those of the control (RCc). Zymosan dissolved in PBS solution was used as a positive control substance which was injected i.p. at 50 μg/g b.w.

Weight-loaded forced swimming performance test – The anti-fatigue effect was estimated by the method of Busnel and Lehmann (1980). ICR mice weighing 25-30 g were administered with test samples orally for 5 days consecutively and 24 hr later, the swimming tests were performed. Mice were divided into three groups of 9 animals each. The swimming test was carried out with a weight attached to the tail of the animal. A weight [(b.w+3 g) × 0.065 g] was attached at 5 cm from the base of the tail. A stainless water tank (divided into 9 separate compartments with 15 cm by 13 cm by 25 cm deep) used was equipped with a circulation pump and a thermostatically controlled heating unit which maintained 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 suspended in gum arabic (5 g/dl) was administered orally as a positive control substance.

Anti-immobilization stress assay – The effects of the antler extracts on immobilization stress were evaluated by estimating various organ weights and the essential constituents of adrenals according to the methods described by Brekhman and Dardymov (1969) with a slight modification. Stress was induced in rats by fixing them on their backs for 48 hr at 45° before sacrifice. Test samples were administered orally for 5 consecutive days. Three hr after the last treatment, animals were sacrificed by cutting carotid artery. Selected internal organs such as adrenal, thymus, spleen and thyroid were dissected and weighed. Ascorbic acid and cholesterol contents in adrenals were determined by the method of Omaye, *et al.* (1972) and Boyer (1993), respectively.

Platelet anti-aggregation screening – Human platelet concentrate was diluted with PBS to obtain platelet rich plasma (PRP) whose platelet count was adjusted to $300\pm50\times10^6$/ml. The turbidimetric measurements of platelet aggregation were performed by the method of Born (1962) with the platelet aggregometer. The lower and the upper limits were set with PRP and with PPP (platelet poor plasma). PRP (450 μl) was pre-incubated for minimum 3 min. prior to the addition of 50 μl of each test solution (or vehicle). Additional incubation at 37°C with stirring at 1000 rpm for 1 min was followed by the addition of collagen to give the final conc. of 2-6 μg/ml for the induction of platelet aggregation. The degree of aggregation of each sample was judged from the initial maximum aggregation and the percent of inhibition was calculated as follows.

$$\% \text{ inhibition} = (a - b)/a \times 100$$

a: degree of aggregation with vehicle
b: degree of aggregation with sample

Anti-thrombotic Assay – Each test sample (or vehicle) was administered orally to ICR mice (20±2 g) for two consecutive days once a day. H_2O ext. was dissolved in distilled water and EtOH ext was prepared as a PVP solution. A mixture of collagen 400 μg plus epinephrine 70 μg/10 ml of saline/kg was injected in one of the tail veins 1 hr. after the second administration of the test sample to induce thrombosis. Each mouse was carefully observed for more than 15 min. whether paralyzed and dead or recovered from the paralysis.

Results and Discussion

Immuno-stimulating activity – The immunostimulating activity of antler extracts was evaluated by the carbon clearance test method established by Wagner *et al.* (1985) and the results were summarized in Table 1. As shown in Table 1, the ethanol

Table 1. Effect of antler extracts on carbon clearance in mice

Treatment		Regression coefficient (RC) of carbon clearance	RCtr/ RCc
Exp.1. (Ethanol ext.)			
Control		0.0216±0.0017[a)]	-
Antler	5 mg/kg/day, p.o.	0.0256±0.0023	1.18[b)]
	10 mg/kg/day, p.o.	0.0342±0.0028	1.58
Zymosan	50 mg/kg, i.p.	0.0350±0.0030	1.62
Exp. 2. (Water ext.)			
Control		0.0208±0.0013	-
Antler	10 mg/kg/day, p.o.	0.0256±0.0010	1.23
Zymosan	50 mg/kg, i.p.	0.0304±0.0016	1.46

[a)] Values are mean S.E. of five mice.
[b)] Assessment of immuno-stimulating potency: RCtr/RCc = <1, not active; <1.5, active; >1.5, very active.

extracts of antler, with 4 consecutive daily oral administrations to mice, exhibited a significant enhancement of carbon clearance activity in a dose response manner. The immuno-stimulating potency expressed as regression coefficient ratio of animal group treated with 5 mg/kg/day was 1.18 which represents a moderate activity. The regression coefficient ratio of animal group treated with 10 mg/kg/day, however, showed 1.58 which represents a very strong activity. This activity was almost equipotent to zymosan, known as a typical phagocytosis enhancer. The water extract of antler, showed a little lower, but a moderate enhancing activity of carbon clearance at a dose of 10 mg/kg/day, p.o.

Antifatigue activity – The antifatigue activity of the antler extracts was evaluated by determining the duration of forced swimming performance in mice (Busnel and Lehmann, 1980) and the results were shown in Fig. 1 and 2. It can be seen that the ethanol extracts of antler, when administered orally in mice once a day for 5 days consecutively, caused a marked prolongation of swimming performance in a good dose response manner. The mean duration of swimming time of the control group was 17.8 min, but those of the groups treated with 50 mg/kg/day, 100 mg/kg/day and 200 mg/kg/day were 48.4 min (171.9% increase) and 69.2 min (288.7% increase), respectively. (Fig. 1) Similar results were obtained also in case of the treatment of water extracts. A marked prolongation of swimming performance could be observed in 50 mg/kg/day, 100 mg/kg/day and 200 mg/kg/day treated groups by 209.6% and 234% increase, respectively.

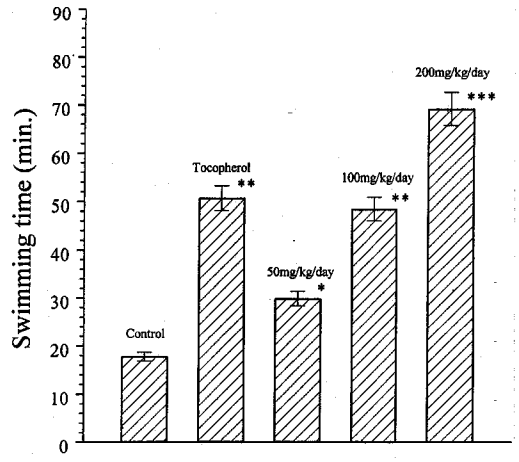

Fig. 1. Effect of ethanol extracts of antler and DL-α-tocopherol on the duration of swimming performance test in mice.
Mice were administered orally with ethanol extracts dissolved in distilled water for 5 days consecutively and the swimming times were evaluated 24hr after the last treatment of test samples. Data are the mean±S.E. of nine mice each.
Significantly different from control group; *p<0.05, **p<0.01, ***p<0.001

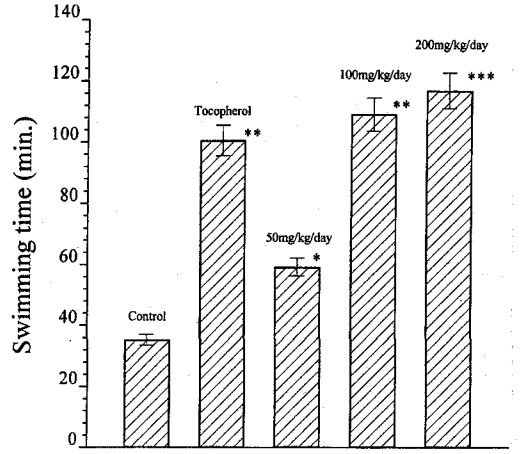

Fig. 2. Effect of water extracts of antler and DL-α-tocopherol on the duration of swimming peformance test in mice.
Mice were administered orally with water extracts dissolved in distilled water for 5 days consecutively and the swimming times were evaluated 24hr after the last treatment of test samples. Data are the mean±S.E. of nine mice each.
Significantly different from control group; *p<0.05, *p<0.01, ***p<0.001

The above results strongly reflect that both ethanol and water extracts of antler possessed a significant

Table 2. Effect of water extracts of antler on some indices in immobilization stress in rats

Treatment	Dose (mg/kg/day, p.o.)	Adrenal (mg)	Thymus (mg)	Spleen (mg)	Thyroid (mg)
Control	-	24.26±1.74	529.1±35.9	567.6±32.1	24.98±3.40
Stress control	-	34.73±2.66[b]	340.1±51.1[b]	315.2±26.4[c]	16.78±1.16[a]
Stress + Antler	50	29.02±2.10[d]	346.9±32.8	365.9±36.1	21.83±0.83[f]
Stress + Antler	200	28.47±1.88[d]	351.3±13.0	403.0±18.5[e]	20.63±0.50[e]

Data represent mean S.E. of five rats in each group.
[a] $P<0.05$, [b] $P<0.02$ and [c] $P<0.01$ as compared to the control.
[d] $P<0.05$, [e] $P<0.01$ and [f] $P<0.001$ as compared to the stress control.

immuno-stimulating and a remarkable antifatigue effect.

Anti-stress effect – The effect of antler extracts on selected internal organs such as adrenal, thymus, spleen and thyroid in rats exposed to immobilization stress were estimated and the results were indicated in Table 2 and 3. It can be seen that immobilization stress caused significant alterations in organ weights i.e., increased adrenal weight, but decreased thymus, spleen and thyroid weights as compared to their corresponding stress controls. Pretreatment with water extract, organ weights significantly reversed towards the normalization at doses of 50 and 200 mg/kg/day. Similar organ weight alterations could be observed in the groups treated with ethanol extracts of antler. It is well known that stress evoke glucocorticoids from adrenal cortex and as a result alters physiological homeostasis. Many stressful conditions are reported to modify host resistance to a variety of physiological disorders (Selye, 1976).

Other organs such as liver, lympocytes including thymus cells, adipose cells, kidney, anterior pituitary, various parts of brain and various related hormones are known to be involved in stress. Brekhman and Dardymov (1969) have shown that immobilization stress caused a significant alteration in weights of adrenal, thymus, spleen and thyroid and some plant extracts possess anti-stress effects. These results are in good agreement with those of our present experiments. Selye (1938) also has established that stress is characterized by the sequence of an alarm reaction and adrenal hypertrophy and depletion of ascorbic acid, cortisol and cholesterol contents are some of predominating features of the alarm reaction. As indicated in Table 4, both water and ethanol extracts at a dose of 200 mg/kg/day, p.o. significantly reversed adrenal ascorbic acid and cholesterol contents as compared to those of stress control groups which were significantly altered by immobilization stress. Bishayee and Chatterjee (1994), however, have shown that in experiment of physical stress induced by 5 hr forced swimming, adrenal weights of stress controls increased but their ascorbic acid contents decreased. Our present results clearly demonstrated that ascorbic acid contents in adrenals of immobilization stress control group were significantly higher than those of non-stress normal control group. This discrepancy is presently unknown.

Anti-thrombotic activity – Platelet aggregation is a crucial factor in the pathogenesis of thrombotic diseases. Because they readily aggregate in response to a variety of endogenous substances and secrete various substances that cause further aggregation, platelets can initiate thrombus formation and precipitate thromboembolism leading to ischemic diseases. In addition, substances secreted from platelets can

Table 3. Effect of ethanol extracts of antler on some indices of immobilization stress in rats

Treatment	Dose (mg/kg/day, p.o.)	Adrenal (mg)	Thymus (mg)	Spleen (mg)	Thyroid (mg)
Control	-	22.35±0.73	249.5±16.8	430.7±15.9	25.78±3.41
Stress control	-	32.11±2.54[b]	194.9±3.40[b]	339.4±8.30[c]	16.03±0.21[a]
Stress + Antler	50	28.38±1.27	240.7±8.00[f]	349.6±11.9	22.21±0.62[e]
Stress + Antler	200	26.76±0.66[d]	239.5±17.4	363.6±10.6	24.06±1.91[e]

Data represent mean S.E. of five rats in each group.
[a] $P<0.05$, [b] $P<0.01$ and [c] $P<0.001$ as compared to the control.
[d] $P<0.05$, [e] $P<0.01$ and [f] $P<0.001$ as compared to the stress control.

Table 4. Effect of antler extracts on ascorbic acid and cholesterol contents in adrenals of rats exposed to immobilization stress

Treatment	Dose (mg/kg/day, p.o.)	Ascorbic acid(mg/g)*		Cholesterol(mg/g)	
		Water ext.	Ethanol ext.	Water ext.	Ethanol ext.
Control	-	0.166±0.026	0.127±0.012	9.77±0.99	10.06±0.73
Stress control	-	0.513±0.040[a)]	0.333±0.004[a)]	2.51±0.36[a)]	2.35±0.32[a)]
Stress + Antler	50	0.403±0.03[b)]	0.314±0.025	6.09±0.87[d)]	6.81±0.58[d)]
Stress + Antler	200	0.389±0.028[c)]	0.254±0.020[b)]	9.22±0.83[e)]	6.21±0.76[d)]

Data represent mean S.E. of five rats in each group. *mg/g of adrenal.
[a)] $P<0.001$ as compared to the control.
[b)] $P<0.05$, [c)] $P<0.02$, [d)] $P<0.01$ and [e)] $P<0.001$ as compared to the stress control.

Table 5. Platelet anti-aggregating effects of extracts of antler

Sample	Conc. (mg/ml)	Inhibition (%)
EtOH ext.	1	84±7
	0.6	58±12
	0.5	46±8
H_2O ext.	1	40±4

*Platelet aggregation was induced with collagen ($2\text{-}6\times10^{-6}$ g/ml)

mediate many other biologic reactions and may also be involved in atherogenesis and other pathologic processes. The extracts of antler were screened for their potential anti-platelet aggregating activities and the results are tabulated in Table 5.

Both EtOH and H_2O extracts showed mild inhibitory activities against collagen-induced platelet aggregation at the concentration of 1 mg/ml. EtOH extract was about 2 times more inhibitory than H_2O extract. The intravenous injection of mixtures of collagen and epinephrine induces platelet aggregation and formation of thrombus leading to the occlusion of the vessels of various organs (Dimino and Silver, 1983). Especially massive occlusion of the pulmonary blood vessels by the formation of platelet thrombi and by vasoconstriction causes short of respiration, paralysis and death. Indeed, within 1 min after the thrombotic challenge, most of the animals became motionless, developed large protruding eyes and began gasping for breath. This was followed by the spasmodic movements, rapid respiration and then death. As shown in the control (H_2O), only 4 mice (15%) recovered from the paralysis within 15 min. Aspirin (50 mg/kg) treatment lowered death in 15 min and increased the recovery from paralysis to 36% indicating significant protective effects of aspirin against the thrombotic challenge. H_2O extract (200 or 500 mg/kg) treated group of mice showed 23-29% recovery in 15 min indicating mild protective effects. Control group of mice treated PVP which is the solubilizing vehicle for EtOH extract showed again 10% recovery within 15 min and 42% of mice were recovered within 15 min from the positive control of PVP-aspirin treated group. EtOH extract (200 or 500 mg/kg) again exhibited protective effects with

Table 6. Anti-thrombotic effects of extracts of antler

Sample (mg/kg)	Total no. of mice tested	Recovered within 6 min	Recovered* within 15 min		Killed within 5 min	
		no.	no.	%	no.	%
Control (H_2O)	27	2	4	15	20	74
H_2O ext (200)	28	2	8	29	14	50
H_2O ext (500)	30	2	7	23	18	60
Aspirin (50)	28	5	10	36	15	54
Control (PVP)	29	0	3	10	21	72
EtOH ext (200)	52	2	17	33	28	54
EtOH ext (500)	48	6	18	38	25	52
Aspirin (50)	26	1	11	42	11	42

*The number of mice recovered within 15 min included the number of mice recovered within 6 min.

the recovery of 33-38% within 15 min of thrombotic challenge. Especially 500 mg/kg of EtOH extract treated group of mice showed higher recovery within 6 min of challenge than aspirin (50 mg/kg) treated group of mice although total recovery within 15 min was slightly lower with EtOH extract (500 mg/kg) treated group than aspirin (50 mg/kg) treated group.

The above *in vitro* and *in vivo* test results are suggestive of mild anti-platelet and anti-thrombotic effects of both water and ethanol extracts of antler.

Conclusion

The present study demonstrated that both ethanol and water extracts of New Zealand antlers have significant immuno-stimulant activity as they enhanced phagocytosis at relatively low dose comparable to those of zymosan, known as a phagocytosis enhancer. The antler extracts also exhibited antifatigue, anti-stress and a weak but significant anti-thrombotic activity. All these findings are indicative of adaptogenic properties of antler and their normalizing effect during stressful condition strongly suggest a state of non-specific increased resistance. Further study with bioassay guided fractionation is required in order to clarify genuine active principles from antlers and ultimately to elucidate the mode of their action more precisely.

Acknowledgement

This work was supported by the research grant from Game Industry Board, New Zealand (1995). The authors would like to thank Ms. Kyung Ah Koo and Ms. Moon Hee Kim for their technical assistance.

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(Accepted February 6, 1999)