Developmental research on moschus-analogue crude drugs

Kiichi KUBO,*[a] Hisashi MATSUDA[a] and Zen-ichi OGITA[b]

[a] *Research Laboratory, Kokando Co., Ltd.*
[b] *Department of Biochemical Pathology, Research Institute for WAKAN-YAKU, Toyama Medical and Pharmaceutical University*

(Received July 17, 1985. Accepted January 10, 1986.)

Abstract

In order to develop new moschus-analogue crude drugs, the chemical components and physiological activities of extracts from reproductive or accessory genital glands obtained from sheep or swine were studied. In order to examine any similarity between extracts from these glands and that of moschus in their physiological effects, electrophoresis and activity measurements were made using the inductive effect on mouse submandibular gland arginine peptidase activity as an index, and the results were subsequently analyzed. Comparison of these data on activity values and zymograms obtained from the electrophoresis measurements revealed that a great similarity in physiological effect existed between sheep testicle extract and moschus extract.

Subsequently studies on similarity in chemical constituents between these two extracts revealed that both extracts contain substances in their polar fractions which induce arginine peptidase activity, and that they also contain various kinds of C_{19} steroid compounds. The polar fraction of the sheep testicle extract and the moschus extract were then subjected to further pharmacological testing for their anti-inflammatory effects. Three methods consisting of the fertilized egg method, the carrageenin paw edema method, and Whittle's method adopted as assays gave similar results for both extracts with regard to anti-inflammatory effect.

These chemical and biological experimental results led us to the conclusion that the polar fraction of the sheep testicle extract is closely akin to moschus extract.

Key words moschus, arginine peptidase (mice submandibular gland), sheep testicle extract, C_{19}-steroid, fertilized hen egg method, carrageenin paw edema method, Whittle's method

Abbreviations BAPNA ; α-N-Benzoyl-D,L-arginine-*p*-nitroanilide, GC ; gas chromatography, Rokushingan ; 六神丸, TLC ; thin-layer chromatography

Introduction

"Moschus," one of the animal folk drugs listed in the Pharmacopoeia of Japan, is a dried preparation of the secretion from the sex glands of *Moschus moschiferus* LINNE (moschus deer),[1] which inhabits Tibet, Yun-nan, and the Himalayas area. Since ancient times moschus has been used as a valuable raw material for the oriental cardiotonic drug, Rokushingan.[2] However, this substance is becoming difficult to obtain

*〒930 富山市梅沢町2-9-1
株式会社広貫堂 研究所 久保喜一
2-9-1, Umezawa-cho, Toyama 930, Japan

Journal of Medical and Pharmaceutical Society for WAKAN-YAKU **3**, 1～9, 1986

because of a decline in the numbers of the moschus deer in recent years along with the enforcement of the Washington Treaty which restricts the amount of captures as a safeguard against probable extinction of the deer. In order to solve this problem, we attempted to develop a new oriental drug possessing biological activities similar to those of moschus by searching for any mammalian source which could be substituted for this rare animal.

As for the physiological activities of moschus, β-adrenergic-potentiating,[3] anti-inflammatory,[4] cardiotonic,[5] and androgenic-like effects[6,7] have been reported. It is also conceivable that additional unknown activities of moschus exist. There are two approaches to the development of an alternative animal source. The first method involves complete analysis and subsequent chemical synthesis of every moschus constituent bringing about its action as a drug. However, it is almost impossible to clarify all of the chemical substances in the secretion which are connected with biological effects. The second method is to analyze the sex glands of animals which are evolutionally close to the moschus deer, and to compare their chemical constituents or biological activities with those of moschus. The latter method will avoid any failure to find unknown but important substances, which would be probable if the first approach were adopted. From an evolutionary viewpoint, it is hardly believable that the moschus deer came into the world suddenly and accidentally. At the same time, no one can confirm the existence of any animal whose sex gland contains similar constituents to those of moschus.

Bearing in mind the above considerations, we picked readily available sheep and swine from the Ariodactyl group of animals to which the moschus deer belongs, and compared the chemical constituents and biological activities of their reproductive or accessory genital glands with those of moschus. We also discussed the possibilities for the development of a new type of moschus-analogue crude drug based on the results obtained.

Materials and Methods

Animals

All experimental animals were obtained from Sankyo Laboservice Co., Tokyo. After feeding the animals for at least one week, they were then subjected to the pharmacological experiment.

Materials

Moschus was purchased from Shibata Co., Osaka. Swine testes was donated from Sasebo Meat Center, and sheep testes was imported from Australia also through Sasebo Meat Center.

Testosterone propionate was obtained from Teikoku Zoki Co., Tokyo. α-N-Benzoyl-D, L-arginine-p-nitroanilide (BAPNA) and cellulose acetate membrane were obtained from Nakarai Co., Kyoto. Tris (hydroxymethyl) aminomethane (Tris), acrylamide, N-1-naphthyl-ethylenediamine dihydrochloride and brilliant blue G were obtained from Wako Pure Chemical Industry Co., Tokyo. Nembutal was obtained from Dainippon Pharmaceutical Co., Tokyo. The other chemicals were obtained from common commercial sources.

Separation of the samples for physiological studies

Crude samples were obtained by direct extraction of crude moschus or testicle extraction of sheep and swine after mincing them with hot acetone for 5 hours. Each extract was dissolved in n-hexane and was extracted with 95% methanol giving n-hexane layer as the non-polar fraction and methanol layer as the polar fraction. The solvent was evaporated in vacuo and the residue was subjected to physiological studies.

Physiological studies for arginine peptidase activity in mice submandibular gland

Male mice 8 weeks of age of the ddY strain weighing around 25 g were used. Male mice were castrated after being anethetized with nembutal (0.02 mg/body weight : intraperitoneally). After one month, mice were divided into twenty-four groups of five animals each. In each group, mice were administered intraperitoneally 20 mg of the crude extracts of moschus, sheep testicle and swine testicle, and 1 mg of testosterone propionate in 0.2 ml of liquid paraffin per day for 2, 3,

4, 6, 8, 10 days, respectively. The submandibular gland was removed from the mice, washed in 0.9 % NaCl solution to remove traces of blood, and then stored at −150 ℃ in liquid nitrogen. These glands were homogenized in 4 g cold deionized water per g of tissue. The tissues were centrifuged at 10000× g for 30 min at 4 °C, and the supernatant was subjected to electrophoretic analysis and determination for arginine peptidase activity.

Electrophoretic analysis for arginine peptidase activity

According to the Kim and Ogita method,[8] electrophoresis was carried out in 7 % polyacrylamide gel for 3 hours at a constant current of 5mA. The gel buffer was 0.05 M Tris-borate buffer (pH 8.8) containing 0.1 % NaCl and electrode buffer was 0.1 M NaOH-borate buffer (pH 6.8). After electrophoresis, a cellulose acetate membrane which had been immersed in substrate solution was placed on the gel and incubated for 30 min at 37 ℃. BAPNA substrate solution was freshly prepared as follows ; 50 mg BAPNA was dissolved in 1 ml of ethylene glycol monomethylether, to which was added 1 ml of 0.1 M phosphate buffer (pH 7.6). After incubation, the cellulose acetate membtane was immersed in a staining solution consisting of 40 ml of 0.1 M Tris-HCl buffer (pH 8.8), 55 mg of N-1-naphthyl -ethylenediamine dihydrochloride, 100 mg of sodium nitrite and 500 mg ammonium sulfamate. After immersion for 5 min, the cellulose acetate membrane was immediately washed in deionized water to remove traces of the staining solution, and then was immersed in 2N HCl solution. Arginine peptidase activities were revealed as red bands on the cellulose acetate membrane.

Determination for arginine peptiaase activity

Modified Riekkinen's method[9] was used as follows ; A substrate solution was prepared from 43.5 mg of BAPNA and 2 ml of dimethylsulfoxide with 50 mM phosphate buffer solution to make a total volume of 100 ml. A mixture of 1 ml of the substrate solution and 1 ml of the sample supernatant was allowed to stand at 37 ℃ for 1 hour. To the solution was added 1 ml of 20 % perchloric acid with stirring, and the resultant solution was centrifuged at 10000× g for 10 min. A solution of 1 ml of the supernatant and 1 ml of 0.2 % sodium nitrite was allowed to stand at 4 ℃ for 10 min and 1 ml of 0.5 % ammonium sulfamate solution was added to it.

After 5 min standing at 4 ℃, 2 ml of 0.5 % N -1-naphthyl-ethylenediamine dihydrochloride was added and the whole was kept at 37 ℃ for 30 min in a dark place. Activity was determined by measuring absorbance at 546 nm.

Thin layer chromatography and gas chromatography analyses of the constituents of the polar fraction of moschus extract and sheep testicle extract

For the thin-layer chromatographical (TLC) analysis, Merck TLC plate (60 F-254) was employed. A 1 : 1 mixture of ethyl acetate and benzene was used as a developing agent. After the development, dil-H_2SO_4 was sprayed and TLC plate was heated to detect the constituents. For the gas chromatographical (GC) analysis, each polar fraction was trimethylsilanized with 20 molar excess of BSA. The glass column (4 mmϕ×1 m) with 1.0 % OV-17 on Gaschrom Q was used at the column temperature 210 °C.

Anti-inflammatory test for the examination of biological similarities

Following three anti-inflammatory tests were conducted and the statistical significances were assessed by Student's t-test.

Fertilized hen egg method : The fertilized hen egg method was carried out according to the method of Otsuka *et al.*[10] Eggs on 6 days of fertilization were sterilized by soaking them in benzalkonium chloride at 37 ± 1 ℃ for 3 days. Then a hole (about 2 cm in diameter) was made on the chorion of the air chamber part, the chorial membrane was cut carefully not to damage the chorioallantois, and a pre-treated filter paper disk was inserted through the hole.

The treatment of the filter paper disk was carried out as follows. Filter paper of 12 cm in diameter was sterilized at an autoclave at 121 ℃ for 20 min, soaked in methanol solution of the prescribed amount of the study agent, and then dried to evaporate the solvent. After the procedures described above the hole was sealed with

cellophane tape and the egg was put in the incubation apparatus. All these operations were performed under sterile conditions. After 4 days of incubation the egg was broken to take out the filter paper disk to which granuloma adhered. The weight of granuloma was calculated by deducting that of the known filter paper disk from that of filter paper disk with granuloma.

Carrageenin-induced paw edema method : Groups of 6～7 Wistar rats (120～140 g) were used. Each study agent (prescribed amount) was suspended in 5 % arabic gum solution and given orally to each rat. One hour later 0.1 ml of 1 % carrageenin was subcutaneously injected at the right hind-paw to induce edema. Then the treated rat paw was put in a container filled with 0.1 % benzalkonium chloride solution and the volume of the overflow was measured at 1, 2, 3, 4, and 5 hours after the injection to examine the severity of edema with the passage of time by use of the rate of increase of edema.

$$\text{rate of increase in edema} = \frac{Ex - Eo}{Eo} \times 100\ (\%)$$

Ec : volume of normal leg before subcutaneous injection at the paw

Ex : volume of the leg with edema after subcutaneous injection

Whittle's method[11] : Male ddY mice (about 20 g) were used. Study agents (prescribed amounts) were suspended in 5 % arabic gum solution and given orally to them. One hour later 2 % brilliant blue solution (0.1 ml/10 g) was injected into the caudal vein and 15 min later 0.7 % acetic acid solution adjusted to pH 3.5 (0.1 mg/kg) was intraperitoneally injected. Forty-five minutes later they were sacrificed by spinal cord dislocation and the abdominal wall was cut to expose the entrails. After washing the entrails with physiological saline, the washings were collected, filtered through glasswool, and diluted with physiological saline to give a final volume of 10 ml. After the addition of 0.1 ml of 0.1 N NaOH to the solution the absorbance was measured at 610 nm to calculate the amount of the pigment leaked by use of calibration curve, and then the rate of inhibition was calculated by comparison with the control values.

Results and Discussion

To make a study of the similarity of effects on physiological activation of enzyme between testicle extracts of swine and sheep and moschus extract an intraperitoneal injection of each extract was given to mice and values of arginine peptidase activity in their submandibular gland were compared. Testosterone was used as the control (Fig. 1). The results showed that the testosterone dose-dependently increased the enzyme activity but the induction of the enzyme activity was weak as seen in the groups receiving moschus extract and animal testicle extracts. Thus, there was a difference in effect on the activity of arginine peptidase in mice submandibular gland between the extracts of native gland and synthesized testosterone. The weak inducing effect on the enzyme activity produced by all the extracts of natural products was supposed to be due to antiandrogen effect.[12]

On the other hand, among the groups receiving the extracts of natural glands arginine activity in both groups receiving sheep testicle extract and moschus extract increased with increasing dosages at lower doses, stayed constant between at the doses of 80 mg (20 mg/day×4) and 160 mg (20 mg/day×8), and then decreased reversely at the high dose of 200 mg (20 mg/day×10). In general, both groups showed a similar pattern. In the group receiving swine testicle extract, however, the activity increased and decreased at the doses of 40 mg (20 mg/day×2) and 60 mg (20 mg/day×3) respectively. Thereafter the activity increased at the dose of 80 mg (20 mg/day×4), and remained unchanged at the range of 80～160 mg (20 mg/day×4～8), and then at the high dose 200 mg (20 mg/day×10) decreased. Namely, the variation pattern of arginine activity in the group receiving swine testicle extract was obviously different from those in both groups receiving moschus extract and sheep testicle extract. Consequently, the effects of sheep testicle extract on the induction and control of arginine peptidase activity was found to be closer to those of mos-

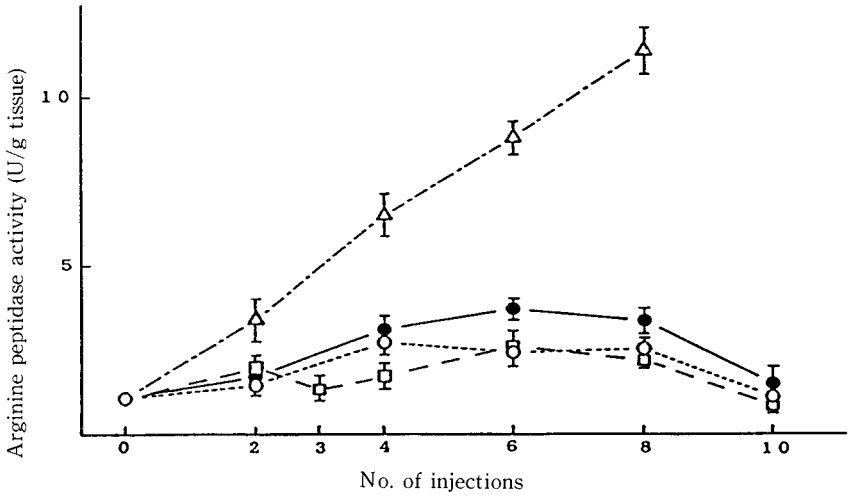

Fig. 1 Change of arginine peptidase activity in submandibular gland of castrated mouse.
The activities are plotted against the No. of injections of testosterone propionate (---△--- 1 mg/ day), moschus extract (—●— 20 mg/day), and testicle extracts of swine (--□-- 20 mg/ day) and sheep (···○··· 20mg/day). Drugs were injected i.p. once a day. Each point shows mean ± S.E. of 5 castrated mouse.

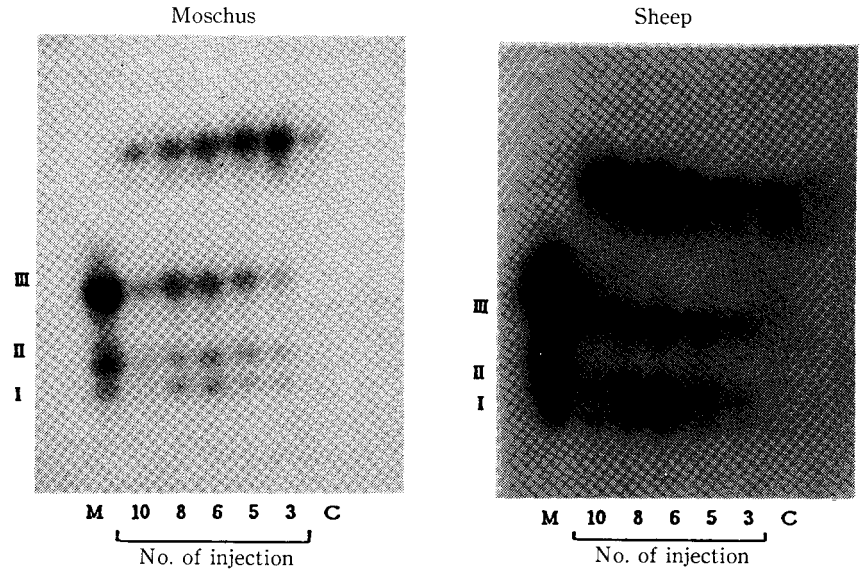

Fig. 2 Change of arginine peptidase zymograms from submandibular gland after the injection of moschus extract and sheep testicle extract into castrated mice.
20 mg of moschus extract or sheep testicle extract in 0.2 ml of liquid paraffin was injected i.p. once a day.
group : 5 castrated mouse.
M : male mice C : castrated mice

chus extract in dose-effect curve compared than those of swine testicle extract.

The effects of moschus extract and sheep testicle extract on arginine peptidase isozyme in mice submandibular gland were compared by means of electrophoresis (Fig. 2). As shown in

the zymograms I, II, and III for arginine peptidase in both groups, the activity of peptidase gradually increased from the start of administration of the extracts up to the dose of 60 mg (20 mg/day×3), reached a plateau between at the doses of 120 mg (20 mg/day×6) and 160 mg (20 mg/day×8), and rapidly decreased at the dose of 200 mg (20 mg/day×10). Namely, the variation pattern of zymogram was extremely similar for both groups.

Hence, further studies were performed on chemical and biological similarities between both extracts, in hope of using sheep testicle extract as a potent alternative to the moschus one. At first, a study was made to obtain a more active fraction from moschus extract and sheep testicle extract which exhibit arginine peptidase activity. Both moschus and sheep testicle extract were fractionated according to the procedure described in "Experimental section" and detection of arginine peptidase activity in each fraction was made by means of electrophoresis. Resultingly each polar fraction in both extracts exhibited the enzyme activity. In 1975, Yoshii *et al.* proved that a group of C_{19} steroids is contained in polar fraction of moschus extract by means of TLC and GC.[13] When we separated the polar fraction of sheep testicle extract by means of TLC, many spots similar to those found in the polar fraction of moschus extract were detected. And then on the GC we also observed many peaks. From these results it was proved that various C_{19} steroidal components were contained in sheep testicle extract as in moschus extract (Fig. 3). Some of

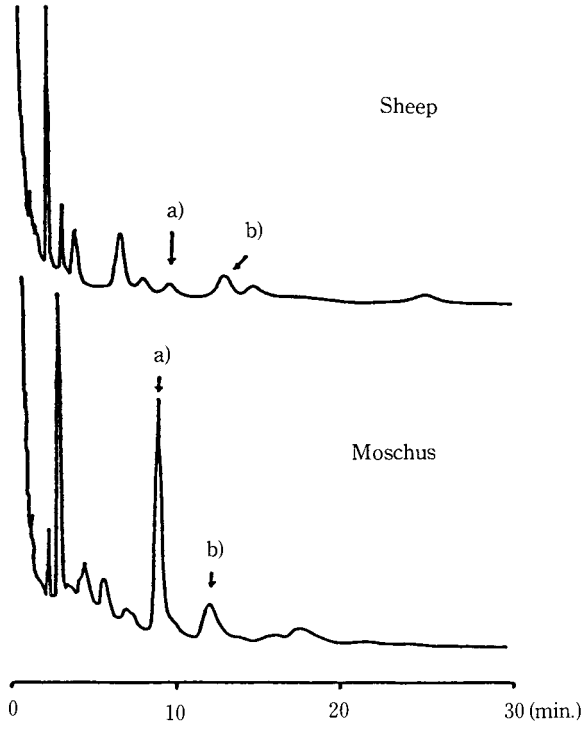

Fig. 3 Gas chromatograms of polar fraction of moschus extract and sheep testicle extract as trimethylsilyl ethers.
a) 5β-androstane-3α, 17α-diol
b) 5β-androstane-3α, 17β-diol

the steroid components contained in moschus extract were also found in sheep testicle extract. From the results given above it is clear that sheep testicle extract is similar to moschus extract in the chemical components of polar fraction.

Next, the anti-inflammatory test was selected as a pharmacological method to examine their biological similarities because pharmacological study on the anti-inflammatory effect of moschus has been made relatively exact. Moreover, progressive stage of inflammation is classified into three stages as follws ; the first stage of increase in capillary permeability, the second stage of leukocytoplania, and the third stage of proliferation. Animal experimental techniques in each stage have already been established. Therefore, the anti-inflammatory test is favorable for the comparative study on the similarity of the effect between these two extracts. So three characteristic methods among the anti-inflammatory tests were used in anticipation of studying on the similarity of the effects between both extracts diversely.

First, the results of the fertilized hen egg test showed that significant inhibition of granulation was observed at the dose of 1.5 μg of moschus extract and at the doses of 10 and 20 μg of sheep testicle extract per filter paper disk, compared with the control (Table I). Dose-response relationship was not observed in both extracts. Therefore, both extracts were found to have similar action.

Table I Anti-inflammatory activity of moschus, sheep testicle extract and hydrocortisone acetate in ferticle egg method.

Drugs	Dose (μg/disc)	Wet weight of granulation tissue (mg/disc) mean ± S.E.	inhibition (%)	Survival ratio of chick embryo
Control		181.0±14.5		9/10
Hydrocortisone acetate	50	108.9± 8.1**	39.8	7/10
Moschus extract	1	135.3±12.2*	25.2	9/10
〃	5	140.5± 8.3**	22.4	10/10
Sheep testicle extract	10	129.2± 8.4**	28.6	9/10
〃	20	128.2±14.7*	29.2	7/10

** : Significantly different from control ($p<0.01$)
* : Significantly different from control ($p<0.05$)

Next, the carrageenin-induced paw edema test was used to examine the inhibitory effect of each extract on edema. Oral administrations of sheep testicle extract (500 mg/kg) and moschus extract (200 mg/kg) produced significant inhibition of edema comparable to that by the known anti-inflammatory agent, phenylbutazone (Fig. 4). In particular the time-effect curve during the period of 5 hours after the start of administration was extremely similar for both extracts. This result also proved that these two extracts were similar in efficacy.

Moreover, Whittle's method was used to test inhibitory effects of both extracts on capillary permeability (Table II). The known agent, aspirin, exerted marked inhibitory effects, while moschus extract exerted poor inhibitory effects. However, interestingly, sheep testicle extract showed a similar tendency to the above method.

There was a little difference in the level of anti-inflammatory effect of moschus extract observed in these experiments and the same could be said of sheep testicle extract. This result suggests that both extracts may have a similar action mechanism. From the results of the chemical and biological experiments described above it can be concluded that polar fraction of sheep testicle extract may be used as a replacement for moschus extract.

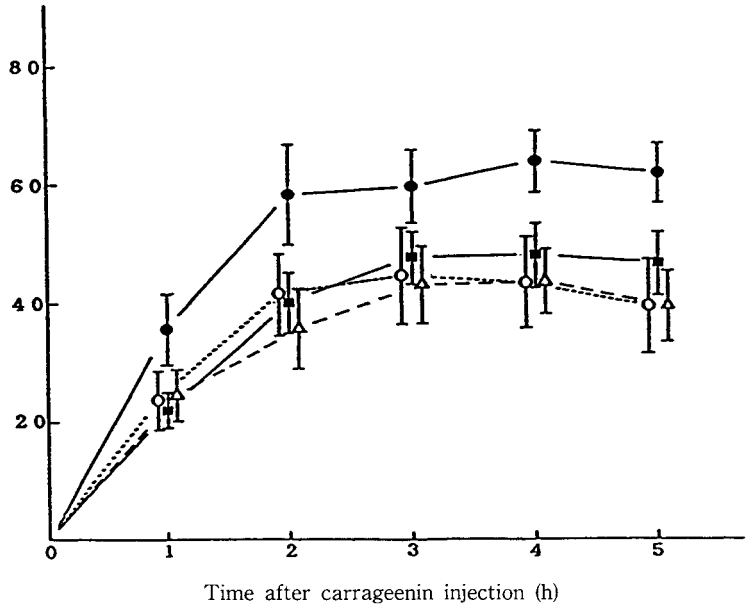

Fig. 4 Effect of moschus extract, sheep testicle extract and phenylbutazone on the swelling of rat hind paws induced by carrageenin.
Control (—●— 5 % arabic gum soln.), moschus extract (···○··· 200 mg/kg), sheep testicle extract (--△-- 500 mg/kg) and phenybutazone (—■— 150 mg/kg) were orally administered 1 h before the injection of carrageenin. Each value represents the mean ± S.E. of 8 animals.

Table II Inhibitory effects of moschus extract, sheep testicle extract and aspirin on capillary permeability.

Drugs	Dose (mg/kg)	Leaked dye in mice (μg)	Inhibition (%)
Control		337±17.6 (9)	
Aspirin	200	126± 8.6** (8)	62.6
Moschus	100	276±20.1* (8)	18.1
Sheep Testicle extract	150	286± 3.6* (8)	15.1

After drugs were orally administered, 2 % brilliant blue soln. was injected the candal vein and 0.7 % acetic acid soln. was injected i.p. Each value represented the mean S.E. for the number of animals given in parentheses.
** : Significantly different from control ($p<0.01$)
* : Significantly different from control ($p<0.05$)

References

1) Moschus. In "The Pharmacopoeia of Japan Ed. 10 (II)," Hirokawa Publishing Co., Tokyo, 1028-1031, 1981
2) Kimura, K. and Osada, E. : On the combined actions of several crude drugs included in "Rokushingan" on Cardiovascular system. *Yakugaku Zasshi* 88, 119-124, 1968
3) Kimura, K., Waki. I. and Ikeda, H. : The potentiation of crude drug "moschus" for catecholamines. *Yakugaku Zasshi* 88, 130-134, 1968
4) Mishira, P.K., Arona, R.B. and Seth, S.D.S. : Anti-inflammatory activity of musk. *J. Pharm. Pharmacol.* 14, 830-831, 1962
5) Takayama, Y. : Über die Wirkung des Moschus, eines Volks-Erregungs Mittels, auf den Kreisauf. *Tohoku J. Exp. Med.* 15, 435-456, 1930

6) Sano, T. : Über die männlichsexualhormonartige Wirkung des Moschus. *Yakugaku Zasshi* **56**, 913-914, 1936
7) Sano, T. : Über die männlichsexualhormonartige Substanz des Moschus. *Yakugaku Zasshi* **56**, 851-862, 1937
8) Kim, Y.K. and Ogita, Z. : Sexual dimorphism of SDS peptide patterns from the submaxillary glands of mice. *J. Exper. Zool.* **218**, 447-453, 1981
9) Riekkinen, P.J., Ekfors, T.O. and Hopsu, V.K. : Purification and characteristics of alkaline protease from rat-submandibular gland. *Biochim. Biophys. Acta* **118**, 604-620, 1966
10) Otsuka, H., Tsukui, M., Matsuoka, T., Goto, M., Fujimura, H., Hiramatsu, Y. and Sawada, T. : Anti-inflammatory screening by fertiled egg method. *Yakugaku Zasshi* **94**, 796-801, 1974
11) Whittle, B.A. : The use of changes in capillary permeability in mice to distinguish between narcotic and nonnarcotic analgesics. *Brit. J. Pharmacol.* **22**, 246-253, 1964
12) Agarwal, M.K. : "Hormone Antagonists," Walter de Gruyter, Berlin & New York, 1982
13) Do, J.C. Kitatsuji, E. and Yoshii, E. : Study on the components of musk. I. Ether soluble components. *Chem. Pharm. Bull.* **23**, 629-635, 1975