Hematological Studies on Naturally Occurring Substances. II.¹⁾ Effects of Animal Crude Drugs on Blood Coagulation and Fibrinolysis Systems

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The effects of 112 extracts prepared from 37 kinds of animal crude drugs and a dog's filaria were investigated on the blood coagulation and fibrinolysis systems *in vitro*. The plasma recalcification time method was employed for the assay of blood coagulation and fibrinolytic activity was tested by the fibrin plate method. It was found that the water extracts of *Bombyx Batryticatus*, *Carpio Fel* and *Holotrichiae Vermiculus* showed potent inhibitory effects on blood coagulation. The methanol extracts of *Phocae Thstis et Penis*, *Scorpion* and *Tabanus* were promotive effect, on the other hand. As regards the fibrinolysis system, the water extracts of *Agkistrodon*, *Lumbricus*, *Hirudo*, *Scolopendra* and *Scorpion* and the methanol extract of *Hirudo* showed a remarkable activity.

Keywords animal crude drug; *Cervi Cauda*; *Hirudo*; blood coagulation accelerator; coagulation inhibitor; fibrinolysis accelerator; fibrinolysis inhibitor; plasma recalcification time method; fibrin plate method

With the aim of finding effective therapeutic drugs for myocardial and cerebral thrombosis from among naturally occurring drugs, Okuyama *et al.* have demonstrated that some Chinese medical prescriptions and their individual components have an effect on human platelet aggregation.²⁻⁶⁾ We have studied the effects on blood coagulation¹⁾ and on fibrinolytic activity⁷⁾ of 120 kinds of oriental plant drugs. Furthermore, we have also examined animal crude drugs employed in oriental medicine for blood activation, screening 112 extracts prepared from 37 kinds of animal crude drugs and a dog's filaria for activity on the blood coagulation and fibrinolysis systems.⁸⁾ The results are presented here.

Experimental

Materials The materials for the tests were supplied by Mikuni Co. (Osaka, Japan).

Preparation of the Test Solution Method I: Each animal crude drug (100 g) was successively extracted with ethyl acetate, methanol and water (each 1 l × 2, 2 h per extraction) under reflux. The ethyl acetate and methanol solutions were evaporated *in vacuo* to dryness and the water solution was lyophilized to yield ethyl acetate (A), methanol (M) and water (W) extracts, respectively.

Method II: The crude drug marked with an asterisk in the table was scorched beforehand in a Pao-Zhi frying pan (Hosei-nabe in Japanese) at 220–300 °C for 30–40 min and treated according to method I.

Each extract (10 mg) was dissolved in 500 μ l of water and filtered with cotton to give a filtrate as the test solution.

Reagents Agarose for electrophoresis (Nacalai Tesque Inc.), fibrinogen (bovine plasma), fibrinogen (bovine plasma plasminogen free) (Nakarai Chemicals Ltd.), thrombin (Mochida Pharmaceutical Co., Ltd.), urokinase (high molecular weight, human urinary) (The Green Cross Corp.), disodium hydrogen phosphate · 12H₂O and potassium dihydrogen phosphate (Kanto Chemical Ltd.) were used. All of them were of fine grade.

Blood Coagulation Test Animal and Blood: Healthy Japanese white male rabbits (body weight; 2.5–4.0 kg) were acclimated at room temperature (22 ± 1 °C) and a relative humidity of 55 ± 5% over 1 week. Fresh blood (9 ml) was carefully collected from an ear artery using a syringe containing 3.8% sodium citrate (1 ml) and was centrifuged at 0 °C 3000 rpm for 10 min to obtain a clear supernatant as platelet-poor plasma (PPP).

Measurement of Blood Coagulation Time: A mixture containing 30 μ l of test solution and 150 μ l of PPP was incubated for 60 s under stirring, then dropped into 0.025 M calcium chloride solution at 37 °C. Coagulation time was evaluated by the use of an automatic recording apparatus (KC-4A, Amelung Co., Ltd.)⁹⁾ based on the viscosity change of the plasma.

Calculation of the Coagulation Time: Each test was repeated at least 3 times on a test animal, and 3–5 animals were employed. The average time

(s) was calculated by applying the following equation: coagulation time (%) = [(coagulation time of each test solution/coagulation time of control) – 1] × 100.

pH Value: The pH values of the test solution and the test solution containing PPP were measured with a pH meter (Compact pH meter C-I; Horiba).

Fibrinolytic Activity Preparation of Fibrin Plate (Modified Noren Method)¹⁰⁾: A 0.3% fibrinogen solution (5 ml) was added to 5 ml of 1% agarose solution, then 0.1 ml of 100 NIH/ml thrombin was added to this mixture with or without plasminogen.

Measurement of Fibrinolytic Activity: The fibrin plate method for screening employed 100 μ l of each test solution (2) with 30 μ l of urokinase solution (600 U/ml), and 10 μ l of aliquots were placed in two 4 mm diameter holes in each two plates. After incubation at 37 °C for 21 h, the diameters of the sample- and control-lysed areas were measured. This procedure was repeated at least 3 times. The value of activity (%) was calculated by use of following equation: fibrinolytic activity (%) = [(lysed area of test solution/lysed area of control) – 1] × 100.

Results and Discussion

Most of the water extracts of animal crude drugs markedly increased blood coagulation time as shown in the table. Among 12 kinds of extract with activity of over 50%, the water extracts of *Bombyx Batryticatus*, *Carpio Fel* and *Holotrichiae Vermiculus* showed particularly strong activity. On the other hand, the methanol extracts of *Phocae Thstis et Penis*, *Scorpion* and *Tabanus* decreased the coagulation time by over 25%. Plant crude drugs were also reported to affect blood coagulation.⁶⁾ Animal crude drugs were generally more potent than plant crude drugs as inhibitors of blood coagulation.

We found that the water extracts of *Agkistrodon*, *Hirudo*, *Lumbricus*, *Scolopendra* and *Scorpion* but not the methanol extract of *Hirudo* strongly increased fibrinolytic activity. The pH values of extracts were around 3–7 and those of the mixtures of extract and plasma were almost neutral.

It is noteworthy that dog's filaria worm, *Filia canina*; *Dirofilaria immitis*, which is parasitic on the dog's heart and prevents thrombus phenomenon in the parasitized area, showed strong activity on the fibrinolysis system. Further study will be necessary to identify the active substance.

Ethyl acetate and methanol extracts tended to inhibit the fibrinolytic activity of urokinase in a plasminogen-containing fibrin plate. The methanol and water extracts of *Cervi Cauda* and the water extracts of *Hirudo* and *Phocae*

TABLE I. Screening of Animal Crude Drugs for Activities on Blood Coagulation and Fibrinolysis Systems

Animal crude drug name	Name of animal source	Extract ^{a)}	Coagulation (%) ^{b)}	Fibrinolysis (%) ^{c)}	Plasmin like activity ^{d)}
Agkistrodon 白花蛇	<i>Agkistrodon acutus</i> GUENTHER	A	—	—	
		M	-5	-3	+
		W	1	114	++
Amydae Carapax 土别甲	<i>Amyda sinensis</i> WEIGMANN	A	22	—	
		M	11	89	++
		W	0	92	++
Antelopis Cornu 羚羊角	<i>Saiga tatarica</i> L.	A	10	8	
		M	9	-62	
		W	3	69	+
Asini Gelatinum 阿膠	<i>Equus asinus</i> L.	A	...	...	
		M	3	-4	
		W	5	82	
Bezoar Bovis 牛黄	<i>Bos taurus</i> L. var. <i>domesticus</i> GMELIN	A	2	7	
		M	5	4	
		W	15	47	
Bombyx Batryticatus 僵蛹	<i>Bombyx mori</i> L.	A	0	50	
		M	25	-1	
		W	107	36	
Bufonis Venenum 蟾酥	<i>Bufo bufo gargarizans</i> CANTOR	A	0	30	
		M	-25	30	
		W	34	88	
Carpio Fel 鲤胆	<i>Cyprinus carpio</i> L.	A	34	-25	
		M	99	96	+
		W	168	96	+
Cervi Cauda 鹿尾	<i>Cervus elaphus</i> L.	A	-3	—	
		M	-2	52	+++
		W	-4	65	+++
Cervi Parvum Cornus 鹿茸	<i>Cervus elaphus</i> L.	A	...	...	
		M	...	...	
		W	5	65	
Cicadae Periostracum 蟬退	<i>Cryptotympana atrata</i> FABR.	A	-10	—	
		M	6	-24	
		W	7	56	
Cordyceps 冬虫夏草	<i>Cordyceps sinensis</i> SACC. & <i>Hepialus armoricanus</i> OBERTHUR	A	22	-48	
		M	28	—	
		W	4	32	
Crinis Carbonisatus 乱髮霜	<i>Homo sapiens</i> L.	A	0	23	
		M	1	-27	
		W	11	-12	
Draconis Os 竜骨	<i>Stegodon orientalis</i> OWEN	A	...	...	
		M	6	-28	
		W	22	57	
Erinacei Corium 刺猬皮	<i>Erinaceus europaeus</i> L.	A	5	-4	
		M	14	-43	++
		W	12	86	++
Eupolyphaga 虻虫	<i>Eupolyphaga sinensis</i> WALKER	A	7	-43	
		M	7	-18	
		W	16	63	
Filia Canina 犬糸状虫 ^{e)}	<i>Dirofilaria immitis</i>	A	...	...	
		M	...	...	
		W	2	98	
Hippocampus 海馬	<i>Hippocampus kelloggi</i> JORDAN et SNYDER	A	83	-6	
		M	52	34	+
		W	14	99	+
Hirudo 水蛭	<i>Whitmania pigra</i> WHITMAN	A	14	35	
		M	2	105	
		W	60	107	+++
Holotrichiae Vermiculus 蟾蜍	<i>Coprides</i> sp.	A	25	32	
		M	13	1	
		W	170	84	
Hominis Placenta 紫苧草	<i>Homo sapiens</i> L.	A	53	-12	
		M	3	76	
		W	7	90	
Hyla 金蛤蟆	<i>Hyla chinensis</i> GUENTHER.	A	37	-54	
		M	4	22	
		W	41	72	+
Lumbricus 地竜	<i>Pheretima asiatica</i> MICHAELSEN	A	11	-18	+
		M	11	75	+
		W	5	101	+

TABLE I. (continued)

Animal crude drug name	Name of animal source	Extract ^{a)}	Coagulation (%) ^{b)}	Fibrinolysis (%) ^{c)}	Plasmin like activity ^{d)}
Mantidis Ootheca 桑螵蛸	<i>Hierodula patellifera</i> SERV.	A	-8	-47	
		M	19	-12	
		W	-4	24	
Moschus 麝香	<i>Moschus moschiferus</i> L.	A	2	35	
		M	12	22	
		W	74	94	
Mylabris 斑蝥	<i>Mylabris sidae</i> FABR.	A	0	-12	
		M	-14	53	+
		W	6	99	+
Phocae Thstis et Penis 海狗腎	<i>Callorhinus ursinus</i> L.	A	13	-26	
		M	-47	49	+
		W	27	86	+++
Ranunculus 御玉杓子	<i>Bufo bufo japonicus</i> SCHLEGEL	A	31	33	
		M	61	85	++
		W	53	96	+
Scolopendra 蜈蚣	<i>Scolopendra morsitans</i> L.	A	5	—	
		M	-29	12	+
		W	55	115	+
Scorpion 全蝎	<i>Buthus martensi</i> KARSCH	A	4	6	
		M	-50	88	+
		W	30	107	+
Sepiae Os 烏賊骨	<i>Sepia esculenta</i> HOYLE	A	49	...	
		M	21	46	
		W	25	91	+
Serpentis Periostracum 蛇退皮	<i>Agkistrodon halys blomhoffii</i> BOIE	A	2	...	
		M	-35	44	
		W	13	47	
Tabanus 虻虫	<i>Tabanus</i> sp.	A	33	-27	
		M	-53	64	+
		W	27	79	+
Troglodyterorum Faeces 五靈脂	<i>Petaurista xanthotis</i> MILNE-EDWARDS	A	3	-37	
		M	36	—	
		W	-3	-3	
Troglodyterorum Faeces 五靈脂 ^{f)}	<i>Petaurista xanthotis</i> MILNE-EDWARDS	A	3	-18	
		M	7	—	
		W	3	5	
Vespae Nidus 露蜂房	<i>Polistes mandarinus</i> SAUSS.	A	8	-17	
		M	7	-11	
		W	24	63	
Vespertilionum Faeces 夜明砂	<i>Vespertilio murinus</i> L.	A	2	50	
		M	2	-12	
		W	28	95	
Xylocopa 鹹竹蜂	<i>Xylocopa dissimilis</i> LEP.	A	10	—	
		M	-30	-31	+
		W	12	43	

a) A, AcOEt extract; M, MeOH extract; W, H₂O extract. b) Coagulation (%)=[(coagulation time of sample/coagulation time of control)-1]×100. c) Fibrinolysis (%)=[(lysed area of sample/lysed area of control)-1]×100. d) Plasmin like activities were compared in terms of lysed area. e) This sample is a dog's filaria worm. f) This animal crude drug was scorched according to the process recommended for preparing the Chinese medicine. —: These extracts inhibited all fibrinolytic activity on the fibrin plate by urokinase. ...: These extracts could not be tested owing to the very low yields.

Thstis et Penis showed plasmin-like action in the fibrin plate without plasminogen.

Generally, it seems that animal crude drugs are more potent than plant crude drugs in their effects on blood coagulation and fibrinolytic activity. The present results justify further studies to identify the active constituents of the extracts.

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