Studies on Alismatis Rhizoma. II. 1) Anti-complementary Activities of Methanol Extract and Terpene Components from Alismatis Rhizoma (Dried Rhizome of Alisma orientale)

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A methanol extract (TMe-ext) from the dried rhizome of Alisma orientale was screened for anti-complementary activity in experimental models. In the animal models, it was found that TMe-ext inhibits zymosan-induced hind paw edema in rats and zymosan-activated rat serum (ZAS)-induced vascular permeability in mice. TMe-ext showed an inhibitory effect on complement-induced hemolysis through both the classical pathway and the alternative pathway. And TMe-ext inhibited hypotonic shock-induced hemolysis, but this effect was weak compared with the anti-complementary activities of TMe-ext. Four triterpenes (alisol A, alisol A monoacetate, alisol B and alisol B monoacetate) isolated from the rhizome also inhibited the complement-induced hemolysis through the classical pathway, but two sesquiterpenes (alisol and alisimoxide) were ineffective. These results indicate that Alismatis Rhizoma shows anti-complementary activity, and its anti-complementary components are partially attributable to the terpene components mentioned above.

Key words Alisma orientale; anti-complementary activity; triterpene; sesquiterpene; hemolysis

Chorei-to (猪苓湯, a prescription composed of five crude drugs: Alismatis Rhizoma, Polypondus, Talcum, Hoelen and Gelatinum) is used for the treatment of cystitis, urolithiasis and urinary disease in the expectation of a diuretic effect and an excrative action of a urinary stone. We have reported that the decoction extract inhibited protein excretion into urine on the anti-glomerular basement membrane (GBM) nephritis caused by GBM antibody, and in addition, we demonstrated an anti-protein extractive action on immune complex (IC) nephritis in both the preventive administration and the therapeutic administration in rats.2)

Among the five drugs in Chorei-to, Alismatis Rhizoma is anticipated to be one of the effective drugs on anti-GBM nephritis and IC nephritis.3) The previous study was also performed to ascertain its effect on type III allergic reactions, which have been referred to as the main factor of IC nephritis, and to explore its active components.3) This paper deals with the inhibitory effect of methanol extract (TMe-ext) from the dried rhizome of Alisma orientale and its anti-allergic components on complement activity, which have been referred to as one factor involved in a type III allergic reaction.

MATERIALS AND METHODS

Materials Alismatis Rhizoma which originated from Alisma orientale Juzep. produced in Fujian province, China, was offered by Nippon Fumatsu Yakuuin Co., Ltd. (Japan). The following drugs were also used: fresh sheep red blood cell (SRBC, Research Foundation for Microbial Disease of Osaka University), hemolysin (Cosmobioc), fresh rabbit blood cell (RrBC, Nikken Bio Medical Laboratory), normal human serum (NHS, Nalgene), gelatin (Wako), barbital, barbitral sodium, ethylene diamine tetraacetic acid disodium salt (EDTA-2Na), ethylene glycol bis (β-aminoethyel)ether)-N,N',N'-tetraacetic acid (EGTA), prednisolone, sodium carbonate (Nacalai Tesque), stigmasterol, and zymosan (Sigma).

Extraction of the Rhizome The crushed rhizomes (1.0 kg) were extracted twice with methanol (10l), under reflux, for 2h. This extract was evaporated under reduced pressure and then freeze-dried to give a methanol extract (TM-ext, 9.8% yield from the rhizome). The contents of the major triterpene constituents, alisol A, A monoacetate, B, and B monoacetate, in this extract were determined according to the method described in a previous paper4) using high performance liquid chromatography (HPLC) [alisol A (0.3652%), alisol A monoacetate (0.3767%), alisol B (0.8267%) and alisol B monoacetate (0.4252%)].

Isolation of the Anti-complementary Components from the Rhizome As shown in Chart 1, the crushed rhizomes (20 kg) were extracted with MeOH three times under reflux for 3h. Evaporation of the solvent from the extract under reduced pressure gave the MeOH extract (1.5 kg). The MeOH extract was partitioned into an EtOAc-H2O mixture, and the H2O-soluble portion was further extracted with 1-BuOH. Removal of the solvent from the EtOAc soluble, 1-BuOH soluble and H2O soluble portions under reduced pressure yielded an EtOAc fraction (240 g), a 1-BuOH fraction (180 g), and a H2O fraction (1.1 kg). The EtOAc fraction (120 g) was subjected to normal or reversed-phase silica gel column chromatography, and two known sesquiterpenes (alisol and alisimoxide) and four known triterpenes (alisol A, alisol A monoacetate, alisol B, and alisol B monoacetate) were isolated.1) These terpenes were also used in this experiment.

Animals Male Sprague-Dawley (SD) strain rats (180—200 g), male ICR strain mice (18—20 g) and male Hartley strain guinea pigs (350—400 g) were provided by SLC (Japan SLC, Japan). They were maintained in an air-conditioned room with lighting from 7 a.m. to 7 p.m. The room temperature (about 23 ºC) and humidity (about 60%) were controlled automatically. A laboratory pellet chow (Labo MR Stock or Labo R Stock, Nihon Nosan Kogyo K. K., Japan) and water were given freely.

Rat Zymosan-Induced Paw Edema Rat zymosan-induced paw edema was induced according to the method of

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Gemmell et al. Male SD strain rats were injected subcutaneously with 0.1 ml of 0.2% zymosan-saline suspension in the hind paw 1 h after the test substances, suspended in 0.5% carboxymethyl cellulose sodium salt (CMC-Na), had been administered orally. The paw volume was measured using a water displacement plethysmometer for up to 5 h at intervals of 1 h. The results were expressed as the percentage of hind paw swelling compared with the initial hind paw volume. Prednisolone was used as a standard drug.

Mouse Zymosan-Activated Serum (ZAS)-Induced Vascular Permeability Male SD strain rats were anesthetized with pentobarbital, blood was withdrawn from the abdominal vein and normal rat serum (NRS) was obtained. After being sterilized, zymosan was suspended in physiological saline at a concentration of 5 mg/ml. One milliliter of the suspension was added to 4 ml of NRS, then the mixture was incubated at 37 °C for 30 min in a water bath, then centrifuged for 20 min at 2000×g. ZAS was obtained.

Male ICR strain mice were injected intravenously with 0.2 ml of 1% Evans blue, and then intraperitoneally with 0.2 ml of ZAS. Thirty minutes later, they were harvested from the peritoneal cavity by rinsing with 10 ml of physiological saline. The absorbance at 616 nm of the solution was measured. Test substances suspended in 0.5% CMC-Na solution were administered orally 1 h before the injection of ZAS. Prednisolone was used as a standard drug.

Complement-Induced Hemolysis through the Classical Pathway This experiment was conducted according to the method of Mori et al. SRBC was washed three times and the cells were added to gelatin veronal buffer (0.1% gelatin, 0.15 M NaCl, 3.12 mM barbitral, 1.82 mM barbitral sodium; GVB<sup>++</sup>). Ten percent SRBC suspension 0.25 ml, GVB<sup>++</sup> 2.5 ml, hemolin 0.25 ml, and complement isolated from fresh serum of guinea pig, 0.5 ml, were added to the test tubes with samples suspended in 5% isopropanol solution, 0.5 ml, and this mixture was incubated at 37 °C for 90 min with frequent shaking. After incubation, the test tubes were centrifuged at 900×g for 10 min, and the optical density of the supernatant was measured at 540 nm. The complete hemolysis was prepared as follows: 10% SRBC suspension 0.25 ml was added to 1% sodium carbonate 3.75 ml. The results were expressed as a percentage of the hemolysis compared with the complete hemolysis. Sodium citrate was used as a standard agent.

Complement-Induced Hemolysis through the Alternative Pathway This experiment was according to the method of Oguni et al. RaRBC was washed three times in gelatin veronal buffer with MgCl<sub>2</sub> and EGTA (0.1% gelatin, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, 0.15 M NaCl, 3.12 mM barbitral, 1.82 mM barbitral sodium; pH 7.4, Mg<sup>2+</sup>–EGTA–GVB). NHS 0.5 ml, Mg<sup>2+</sup>–EGTA–GVB 0.5 ml with test samples suspended in 5% isopropanol solution 0.5 ml, were added to the test tubes, then preincubated at 37 °C for 30 min with frequent shaking. Then, a 5×10<sup>10</sup> cells/ml RaRBC suspension 0.1 ml was added to the above solution and incubated at 37 °C for 40 min. The reaction was stopped by the addition of 0.01 M EDTA-2Na-saline solution 2.5 ml, and the new mixture was centrifuged at 900×g for 10 min. The optical density of the supernatant was measured at 414 nm. The complete hemolysis was prepared as follows: RaRBC suspension 0.1 ml was added to 0.1% sodium carbonate 4.0 ml. The results were expressed as a percentage of the hemolysis compared with the complete hemolysis. Stigmasterol was used as a standard agent.

Hypotonic Shock-Induced Hemolysis SRBC was washed three times and the cells were added to GVB<sup>++</sup>. A ten percent SRBC suspension 0.25 ml and 60 mM NaCl solution 3.25 ml, were added to the test tube with samples suspended in GVB<sup>++</sup> 0.5 ml, and the mixture was incubated at 37 °C for 60 min with frequent shaking. After incubation, the test tubes were centrifuged at 900×g for 10 min, and the optical density of the supernatant was measured at 540 nm. The complete hemolysis was prepared as follows: 10% SRBC suspension 0.25 ml was added to 0.1% sodium carbonate 3.75 ml. The results were expressed as a percentage of the hemolysis compared with the complete hemolysis. Sodium citrate was used as a standard agent.

Statistical Analysis The experimental data were tested for statistically significant differences by means of the Bonferroni/Dunn test.

RESULTS

Rat Zymosan-Induced Paw Edema As shown in Fig. 1, 1Me-ext (200 mg/kg, p.o.) had a significant inhibitory effect on the paw edema at 1, 2, 4 and 5 h after the injection of
zynosan. A positive control agent, prednisolone 25 mg/kg, p.o., also reduced the paw edema.

**Mouse ZAS-Induced Vascular Permeability** As shown in Fig. 2, TMe-ext at an oral dose of 200 mg/kg inhibited mouse peritonitis induced by ZAS. A standard agent, prednisolone (10 mg/kg, p.o.), significantly inhibited the peritonitis.

**Complement-Induced Hemolysis through the Classical Pathway** As shown in Fig. 3, the hemolysis percentage of the complement-induced hemolysis through the classical pathway was 66.9 ± 0.1% in the control group. TMe-ext (at a concentration of 50—200 μg/ml) exhibited a strong inhibitory effect on this hemolysis. The standard agent, sodium citrate (500 μg/ml), also showed inhibition.

Among three fractions from TMe-ext, the EtOAc soluble portion inhibited the complement-induced hemolysis through the classical pathway. The water and 1-BuOH soluble portions did not (Fig. 4).

Four triterpenes, alisol A, alisol A monoacetate, alisol B and alisol B monoacetate, isolated from the EtOAc soluble portion exhibited inhibitory effects on this hemolysis, but two sesquiterpenes, alismol and alismoxide were ineffective (Fig. 5).

**Complement-Induced Hemolysis through the Alternative Pathway** As shown in Fig. 6, the hemolysis percentage of complementary hemolysis through the alternative pathway was 44.3 ± 0.9% in the control group. TMe-ext (at concentra-
Fig. 6. Effects of TMe-ext from Alismatis Rhizoma and Stigmastanol on Complement-Induced Hemolysis through the Alternative Pathway

The mixture of TMe-ext or stigmastanol and buffer were added to complement isolated from human. After 30 min, RBC was added to the solution, followed by incubation for 40 min. The reaction was stopped by EDTA 2Na solution, then the mixture was centrifuged. The absorbance at 540 nm of the supernatant was measured. Each value represents the mean±S.E. of 3 test tubes. Significantly different from control group, *p<0.05, **p<0.01.

Fig. 7. Effects of TMe-ext from Alismatis Rhizoma and Sodium Citrate on Hypotonic Shock-Induced Hemolysis

The mixture of TMe-ext or sodium citrate and SRBC solution was added to sodium chloride. After incubation for 60 min, the reaction was stopped in cold water, then the mixture was centrifuged. The absorbance at 540 nm of the supernatant was measured. Each value represents the mean±S.E. of 3 test tubes. Significantly different from control group, *p<0.01.

Hypotonic Shock-Induced Hemolysis As shown in Fig. 7, the hemolysis percentage of the hypotonic shock-induced hemolysis was 87.5±1.1% in the control group. TMe-ext (at concentration of 50—200 µg/ml) exhibited an inhibitory effect on this hemolysis. Sodium citrate (500 µg/ml) also inhibited this hemolysis.

DISCUSSION

This study aims to elucidate the inhibitory effect of Alismatis Rhizoma on complement activity, which is referred to as the main factor of a type III allergic reaction.

It is known that the complement system has twoactivation routes, the classical pathway and the alternative pathway. The classical pathway is activated by an antigen–antibody complex. On the other hand, the alternative pathway is activated by endotoxin, etc. At last, the two pathways produce a C5b-9 complex (membrane attack complex; MAC), which is attached to a cell membrane in bacteria, etc.11) These routes generate the complement-derived chemotactic factors C5a and C3a, which cause an accumulation of polymorphonuclear leukocytes (PMNs) at the inflammatory site.12) These chemical mediators enhance vascular permeability, promote the wandering of plasmic components and neurophile leukocytes, then amplify the response.13)

First, the effect of TMe-ext from Alismatis Rhizoma on the complement system was investigated in two animal models. TMe-ext showed an inhibitory effect on the paw swelling in rats induced by zymosan, which activated the complement system through the alternative pathway.14) TMe-ext also inhibited the vascular permeability in mice induced by ZAS containing MAC. According to these results and a previous paper,15) it was suggested that TMe-ext inhibits not only the classical pathway, but also the alternative pathway in animal models. The mechanism of TMe-ext may be related to either its inhibitory effect on the formation of MAC in complement activation, or its directly antagonistic effect on MAC activity.

In vitro, the effect of TMe-ext on complement related hemolysis was observed. TMe-ext showed inhibitory activity on hemolysis due to complement activation through either the classical pathway or alternative pathway. Meanwhile, TMe-ext also suppressed the hemolysis caused by hypotonic shock, but its potency was lower than that in complement-induced hemolysis. It was concluded that the inhibitory activity of TMe-ext on the hemolysis accompanied by complement activation was strong and specific, especially when it was activated through the classical pathway.

TMe-ext was fractionated, as shown in Chart 1. On complement-induced hemolysis through the classical pathway, the EtOAc-soluble fraction exhibited a superior inhibitory effect compared with that of TMe-ext. Four triterpenes (alisol A, alisol A monoacetate, alisol B and alisol B monoacetate) isolated from the EtOAc fraction using silica gel column chromatography also showed the inhibitory effect on this reaction hemolysis, whereas two sesquiterpenes (alisomol and alisoxide) were ineffective. Although Tomoda et al.15)-17) have reported that the polysaccharides (alisman PII and PIII) from Alismatis Rhizoma possess anti-complementary activities, these activities seem to be weaker than that of the four triterpenes mentioned above. These triterpenes can be considered the major active principles of Alismatis Rhizoma responsible for its anti-complementary activity.

The present study clearly demonstrates that TMe-ext has an inhibitory effect on complement activity, which is referred to as one factor contributing to type III allergic reactions. Furthermore, alisol A, alisol A monoacetate, alisol B and alisol B monoacetate, isolated from its lipophilic fraction, were thought to be the active components.

In addition, on clinical application of Alismatis Rhizoma for the treatment of diseases related to complement, it is desirable that the preparations be in pill or powder form rather than a decoction.

REFERENCES