Studies on Alismatis Rhizoma. I. Anti-allergic Effects of Methanol Extract and Six Terpene Components from Alismatis Rhizoma (Dried Rhizome of *Alisma orientale*)

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Methanol and aqueous extracts (TMe-ext and TAq-ext) from dried rhizomes of *Alisma orientale* have been screened for activity in experimental models of type I-IV allergies. In the type III allergic model, TMe-ext at oral doses of 50, 200 mg/kg showed an inhibitory effect on the direct passive Arthus reaction (DPAR) in rats, while TAq-ext did not. Four triterpenes (alisol A, alisol B, alisol A monoacetate and alisol B monoacetate) and two sesquiterpenes (alismol and alismoxide) isolated from TMe-ext also exhibited this inhibitory effect. In a type I allergic model, TMe-ext inhibited 48-h homologous passive cutaneous anaphylaxis (PCA) in rats. In a type II allergic model, it was found that TMe-ext inhibits reversed cutaneous anaphylaxis (RCA) in rats. Furthermore, in a type IV allergic model, TMe-ext had an inhibitory effect on the induction phase in picryl chloride-induced contact dermatitis (PC-CD) in mice. These results indicate that Alismatis Rhizoma not only inhibits antibody-mediated allergic reactions but also influences cell reactions and should be recognized as a material for the treatment of allergic reactions, and the anti-type III allergic components are partially attributable to the terpenes mentioned above.

Key words *Alisma orientale*; Chinese traditional crude drug; type I—IV allergic reactions; triterpene; sesquiterpene

Choreito (猪苓湯, a prescription composed of five crude drugs : Alismatis Rhizoma, Polyporus, Kadinum, Hoelen and Gelatinum) is used for the treatment of cystitis, urolithiasis and urinary disease in the expectation of a diuretic effect and an excretive action of a urinary stone. We have reported that the decoction extract inhibited protein excretion into urine, increases in total cholesterol and urea nitrogen level in serum on anti-glomerular basement membrane (GBM) nephritis caused by GBM antibody at preventive administration in rats, and in addition, demonstrated an anti-protein extractive action on immune complex (IC) nephritis in both the preventive administration and the therapeutical administration in rats.[1)]

Among the five drugs in Choreito, Alismatis Rhizoma is anticipated to be one of the effective drugs on anti-GBM nephritis and IC nephritis.[2)] The present study was performed to ascertain its effect on type III allergic reactions, which are referred to as the main factor of IC nephritis, and to explore its active components. The effects of a methanol extract (TMe-ext) from Alismatis Rhizoma on type I, II and IV allergic reactions were further investigated.

MATERIALS AND METHODS

Materials Alismatis Rhizoma (originated from *Alisma orientale* JUZEP.) from Nippon Funmatsu Yakuhin Co., Ltd. (Japan) were used in this study. The following drugs were also used : aluminum hydroxide gel (Maruishi), egg albumin (EWA, Sigma), an inactive bacterial suspension of Bordetella pertussis (Wako), disodium cromoglycate (DSCG, Funakoshi Yakuhin), picryl chloride, dexamethasone, prednisolone (Nacalai Tesque) and Freund's complete adjuvant (Difco).

Extraction of the Rhizome The crushed rhizomes (1.0 kg) were extracted twice with methanol (10 l), under reflux, for 2 h. Additional crushed rhizomes (1.0 kg) were extracted twice with hot water (90 to 100 °C). These extracts were evaporated under reduced pressure and then freeze-dried to give a methanol extract (TMe-ext, 9.8% yield from the rhizome) or an aqueous extract (TAq-ext, 22.8% yield). The contents of the major triterpene constituents, alisols A, A monoacetate, B, and B monoacetate, in these extracts were determined according to the method described in a previous paper[3)] using high performance liquid chromatography (HPLC) [TMe-ext: alisol A (0.3652%), alisol A monoacetate (0.3767%), alisol B (0.8267%), alisol B monoacetate (0.4252%); TAq-ext: alisol A (0.0012%), alisol A monoacetate (0.0008%), alisol B (0.0010%), alisol B monoacetate (0.0003%)].

Isolation of the Anti-type III Allergic Components from the Rhizome As shown in Chart 1, the crushed rhizomes (20 kg) were extracted with MeOH three times under re-

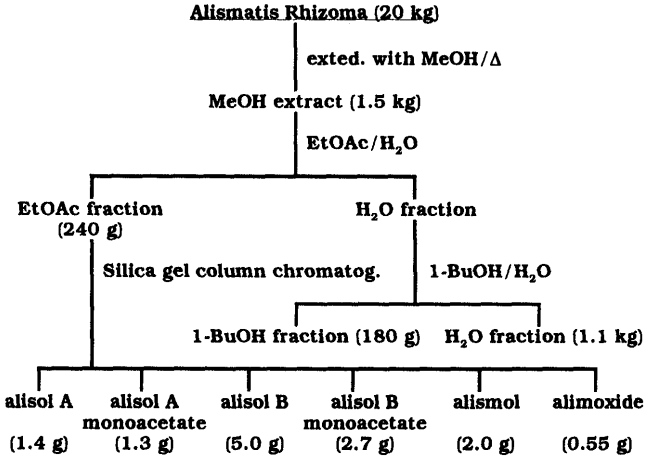

Chart 1. Fractionation and Isolation of Six Terpenes from Alismatis Rhizoma

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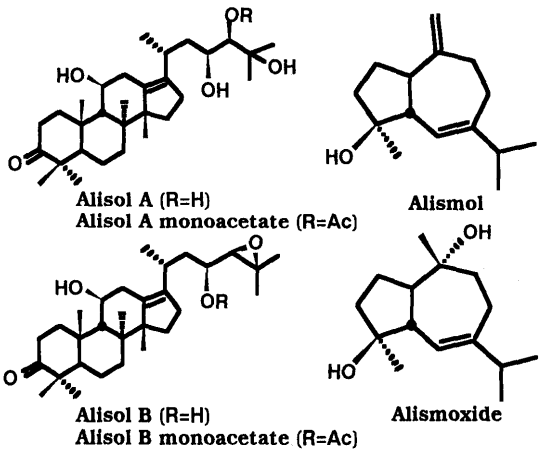

Fig. 1. Structures of Six Terpenes Isolated from Alismatis Rhizoma

flux for 3 h. 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–H_2O mixture, and the H_2O-soluble portion was further extracted with 1-BuOH. Removal of the solvent from the EtOAc soluble, 1-BuOH soluble, and H_2O soluble portions under reduced pressure yielded an EtOAc fraction (240 g), a 1-BuOH fraction (180 g), and a H_2O fraction (1.1 kg). The EtOAc fraction (120 g) was subjected to normal-phase silica gel column chromatography [BW-200 (Fuji Silysia Chemical, Ltd.), $CHCl_3$, and $CHCl_3$–MeOH (50 : 1—10 : 1)] to furnish five fractions. Removal of the solvent under reduced pressure gave fr. 1 (12 g), fr. 2 (33 g), fr. 3 (52 g), fr. 4 (7 g), and fr. 5 (8 g). Fraction 2 was purified by repeated normal-phase silica gel column chromatography [i) *n*-hexane–acetone (7 : 1), ii) benzene–acetone (5 : 1)] to give alismol[4,5] (2.0 g). Normal-phase silica gel column chromatography [$CHCl_3$–MeOH (20 : 1)] of fraction 3, followed by reversed-phase silica gel column chromatography [Chromatorex DM1020T (Fuji Silysia Chemical Ltd.), 50% aqueous methanol], afforded alismoxide[4,5] (0.55 g), alisol A monoacetate[6,7] (1.3 g), alisol B[6,7] (5.0 g), and alisol B monoacetate[6,7] (2.7 g). Fraction 4 was subjected to reversed-phase silica gel column chromatography (50% aqueous methanol) and subsequent normal-phase silica gel column chromatography [benzene–acetone (5 : 1)] to give alisol A[6,7] (1.4 g). Two known sesquiterpenes, alismol, and alismoxide, and four known triterpenes, alisol A, A monoacetate, B, and B monoacetate, were identified by TLC, $[\alpha]_D$, IR, ^{1}H-NMR, and ^{13}C-NMR data comparisons with authentic samples.[4-7] The structures are shown in Fig. 1.

Animals Male Slc:Sprague-Dawley (SD) strain rats (150—170 g), male Slc:Wistar strain rats (150—170, 180—200 g), female Slc:ICR strain mice (30—32 g), male Slc:Hartley strain guinea pigs (250—300 g) and male Slc:JW strain rabbit (2.0—2.5 kg) were used. They were maintained in an air-conditioned room with lighting from 7 a.m. to 7 p.m. The room temperature (about 23 °C) 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.

Direct Passive Arthus Reaction (DPAR, Type III Allergic Model) Rabbit anti-EWA serum was prepared according to the method of Koda *et al.*[8]; briefly, 10 ml of Freund's complete adjuvant was mixed with 10 ml of saline containing 20 mg of EWA. One ml of this mixture was intramuscularly injected into rabbits weighing 2.0—2.5 kg once weekly for 4 weeks. One week after the final injection, the rabbits were anesthetized with pentobarbital, blood was withdrawn from the carotid arteries and rabbit anti-EWA serum was obtained. The anti-EWA IgG antibody (1 : 1024) was determined by PCA in guinea pigs. The PCA titer was expressed as the highest dilution causing a lesion more than 5 mm in diameter.

Rabbit anti-EWA serum (0.5 ml/150 g body weight) was intravenously injected into male SD strain rats weighing 150—170 g *via* the tail vein. Thirty min later, 0.1 ml of saline containing 0.025 mg of EWA was intracutaneously injected into the right hind paw of the rats to induce an Arthus reaction.[9,10] The hind paw volume was measured from 1 to 5 h after the challenge of EWA at 1 h intervals, and the results were expressed as a percentage of the swelling compared with the initial hind paw volume. Test substances suspended in 0.5% carboxymethyl cellulose sodium salt (CMC·Na) solution were administered orally 1 h before the challenge. Prednisolone was used as a standard drug.

Forty-Eight-h Homologous Passive Cutaneous Anaphylaxis (PCA, Type I Allergic Model) Rat anti-EWA serum containing IgE was prepared by the method of Stotland and Share[11] in male Wistar strain rats weighing 180 to 200 g. The rats were immunized with 0.5 ml of suspension containing 1.0 mg of EWA, 20 mg of aluminum hydroxide gel (s.c.) and 1 ml of inactive bacterial suspension of Bordetella pertussis (2×10^{10} cells/ml, i.p.), simultaneously. Seven days later, the rats were immunized again following the same procedure cited above. Fourteen days later, the rats were anesthetized with pentobarbital, blood was withdrawn from the carotid arteries and rat anti-EWA serum was obtained. Serum was stored at −80 °C until use. The anti-EWA IgE antibody (1 : 32) was determined by PCA in Wistar rats. The PCA titer was expressed as the highest dilution causing a lesion more than 5 mm in diameter.

Antiserum diluted 8-fold or 16-fold with saline was injected into 2 sites, respectively, on the shaved dorsal skin of male Wistar strain rats weighing 180 to 200 g, intradermally in a 0.05 ml dose. Forty-eight h after sensitization, the rats were challenged with 0.5 ml of saline containing 10 mg of EWA and 5 mg of Evans blue *via* the tail vein. Thirty min later, the rats were sacrificed, the dorsal skin was removed for measurement of the blue area. The amount of leaked dye was then determined colorimetrically after incubation with 1.0 N KOH, followed by extraction with a mixture of acetone and phosphoric acid based on the method of Katayama *et al.*[12] TMe-ext suspended in 0.5% CMC·Na solution was administered orally 1 h before the challenge. DSCG dissolved in saline was intravenously administered 1 min before the challenge.

Reversed Cutaneous Anaphylaxis (RCA, Type II Allergic Model) Rabbit anti-rat serum was prepared according

to the method of Unger *et al.*[13]; briefly, 1 ml of normal rat serum was intravenously injected into rabbits weighing 2.0—2.5 kg, and this was repeated 10 times at 1-d intervals. One day after the final immunization, the rabbits were anesthetized with pentobarbital, blood was withdrawn from the carotid arteries and antiserum obtained. The serum was stored at −80 °C until use.

Rabbit anti-rat serum (0.05 ml) containing 1% Evans blue or the same volume of saline containing 1% Evans blue was injected into the dorsal skin of male Wistar strain rats weighing 150—170 g. Two hours later, the rats were sacrificed and the dorsal skin was removed. The inflamed areas were cut out with a leather punch (12 mm in diameter).[13] The swelling percentage was expressed by the following equation: swelling $\% = (W_i - W_s)/W_s \times 100$, where W_i is the weight of the inflamed site and W_s is the weight of the saline-injected site. TMe-ext suspended in 0.5% CMC·Na solution was administered orally 1 h before the injection of antiserum. Dexamethasone was used as a standard drug.

Picryl Chloride-Induced Contact Dermatitis (PC-CD, Type IV Allergic Model) The procedure was in accordance with the method of Asherson and Ptak.[14] Female ICR strain mice weighing 30—32 g were sensitized by applying 0.1 ml of 7% picryl chloride solution in ethanol to the shaved abdomen. After a 6-d sensitization period, the mice were challenged by painting the inside of the right ear with 0.02 ml of 1% picryl chloride solution in olive oil to induce PC-CD. In research on the induction phase of PC-CD, the ear thickness was measured with a dial thickness gage (Mitsutoyo, Japan) before and 24 h after the challenge, and the difference in thickness was calculated. TMe-ext suspended in 0.5% CMC·Na solution was administered orally from day −1 to day 6 after immunization. Prednisolone suspended in 0.5% CMC·Na solution was also administered orally from day 0 to day 6 after immunization.

In the study of the effector phase of PC-CD, mice with a certain percentage of ear swelling after sensitization and challenge were chosen; 3 d thereafter they were subjected to sensitization and challenge again by the same procedure, and the percentage of ear swelling was again determined. TMe-ext suspended in 0.5% CMC·Na solution was administered orally before and 16 h after the challenge. Prednisolone suspended in 0.5% CMC·Na solution was administered orally 16 h after the challenge.

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

RESULTS

Effects of TMe-ext and TAq-ext on DPAR As shown in Fig. 2, TMe-ext (50, 200 mg/kg) produced a significant inhibition of the paw swelling induced by DPAR from 1 to 5 h after the injection of antigen in rats. By contrast, TAq-ext was ineffective. The control agent, prednisolone (25 mg/kg), also showed inhibition.

Effects of Three Fractions from TMe-ext on DPAR EtOAc soluble fraction (50 mg/kg) inhibited the paw swelling induced by DPAR from 1 to 3 h after injection of the antigen in rats, but water and 1-BuOH soluble fractions did not (data not shown).

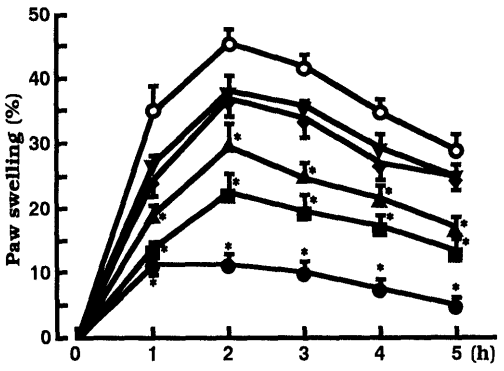

Fig. 2. Effects of Methanol and Aqueous Extracts (TMe-ext and TAq-ext) from Alismatis Rhizoma and Prednisolone on Direct Passive Arthus Reaction (DPAR) in Rats

TMe-ext, TAq-ext or prednisolone suspended in 0.5% CMC·Na were orally administered 30 min before the intravenous injection of an antibody. Thirty min after the injection of the antibody, an antigen was intracutaneously injected into the right hind paw of the rats. The paw volume was measured, and the swelling percentage was calculated. Control was orally administered 0.5% CMC·Na alone. Each point represents the mean ± S.E. of 8 rats. Significantly different from the control group, $*p < 0.01$. ○, control; ▲, TMe-ext 50 mg/kg; ■, TMe-ext 200 mg/kg; ▼, TAq-ext 50 mg/kg; ◆, TAq-ext 200 mg/kg; ●, prednisolone 25 mg/kg.

Effects of Six Terpenes on DPAR Six terpenes, alisol A, alisol B, alisol A monoacetate, alisol B monoacetate, alismol and alismoxide (0.05, 0.20 mmol/kg) exhibited inhibitory effects on the paw swelling induced by DPAR from 1 to 5 h after injection of the antigen in rats (Fig. 3). Among these terpenes, alisol B monoacetate exhibited the greatest inhibition.

Effect of TMe-ext on 48-h Homologous PCA The total dye amount which leaked into the skin was 9.6 ± 0.9 μg/site in the control group. When TMe-ext (200 mg/kg) was administered to rats, the dye leakage was inhibited, as shown in Fig. 4. The control agent, DSCG (5 mg/kg), also inhibited this leakage.

Effect of TMe-ext on RCA As shown in Fig. 5, TMe-ext (50, 200 mg/kg) inhibited the skin swelling induced by RCA in rats. The control agent, dexamethasone (5 mg/kg), also inhibited this increase.

Effect of TMe-ext on PC-CD The effects of TMe-ext and the control agent, prednisolone, on the induction phase of PC-CD in mice are shown in Fig. 6. TMe-ext (200 mg/kg) significantly inhibited ear swelling 24 h after the challenge. Prednisolone (10 mg/kg) also showed inhibition.

As shown in Fig. 7, TMe-ext (50 or 200 mg/kg) did not inhibit the ear swelling of the effector phase of PC-CD, while prednisolone (20 mg/kg) did.

DISCUSSION

This study was performed in order to elucidate the effects of Alismatis Rhizoma on various anti-allergic reactions, centering on the type III allergic reaction.

A methanol extract (TMe-ext) from Alismatis Rhizoma showed an inhibitory effect on paw swelling induced by DPAR, a typical type III allergic model in rat, whereas an aqueous extract was ineffective.

The Arthus reaction is induced by deposits of an immune

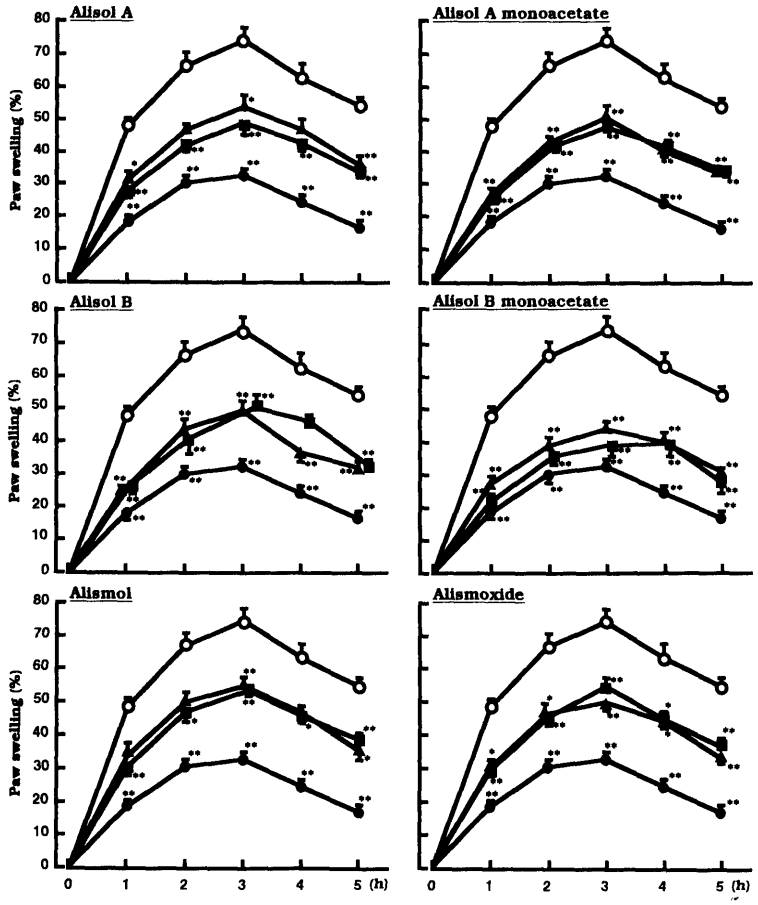

Fig. 3. Effects of Six Terpenes Isolated from Alismatis Rhizoma and Prednisolone on Direct Passive Arthus Reaction (DPAR) in Rats

Arthus reaction was induced by the method in the legend to Fig. 2. Six terpenes isolated from Alismatis Rhizoma or prednisolone suspended in 0.5% CMC·Na were orally administered 30 min before the intravenous injection of an antibody. The paw volume was measured, and swelling percentage was calculated. Control group was orally administered 0.5% CMC·Na alone. Each point represents the mean ± S.E. of 8 rats. Significantly different from the control group, $*p<0.05$, $**p<0.01$. ○, control; ▲, samples 0.05 mmol/kg; ■, samples 0.20 mmol/kg; ●, prednisolone 0.07 mmol/kg (=25 mg/kg).

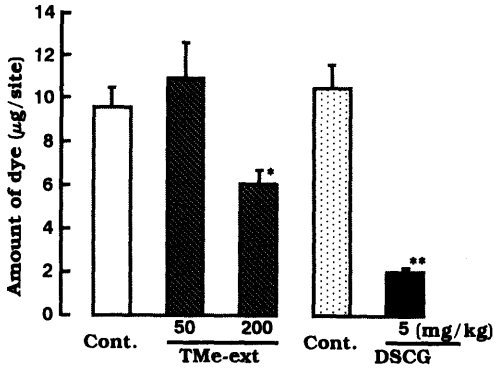

Fig. 4. Effects of Methanol Extract (TMe-ext) from Alismatis Rhizoma and Disodium Cromoglycate (DSCG) on 48-h Homologous Passive Cutaneous Anaphylaxis (PCA) in Rats

Antibody was mediated to rats. After 48 h, TMe-ext suspended in 0.5% CMC·Na was orally administered 1 h before the challenge with an antigen. DSCG dissolved in saline was intravenously administered 1 min before the challenge with an antigen. Control was orally administered 0.5% CMC·Na or intravenously administered saline alone. Each column represents the mean ± S.E. of 7 rats. Significantly different from the control group, $*p<0.05$, $**p<0.01$.

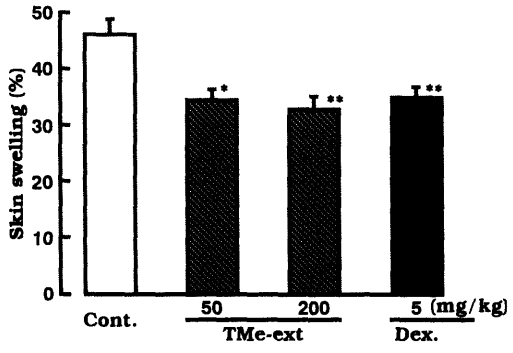

Fig. 5. Effects of Methanol Extract (TMe-ext) from Alismatis Rhizoma and Dexamethasone (Dex.) on Reversed Cutaneous Anaphylaxis (RCA) in Rats

TMe-ext or dexamethasone suspended in 0.5% CMC·Na was orally administered 1 h before the injection of rabbit anti-rat serum. Control was orally administered 0.5% CMC·Na alone. Each column represents the mean ± S.E. of 7 rats. Significantly different from the control group, $*p<0.05$, $**p<0.01$.

complex in the vascular wall. The deposition is followed by the activation of the components of a complement, resulting in the generation of complement-derived chemotactic factor C5a and C3a. These factors cause the accumulation of polymorphonuclear leukocytes at the inflammatory site. These chemical mediators enhance vascular permeability, promote the wandering of plasmic components and neurophilic leukocytes, and then amplify the response.

TMe-ext was fractionated, as shown in Chart 1. An

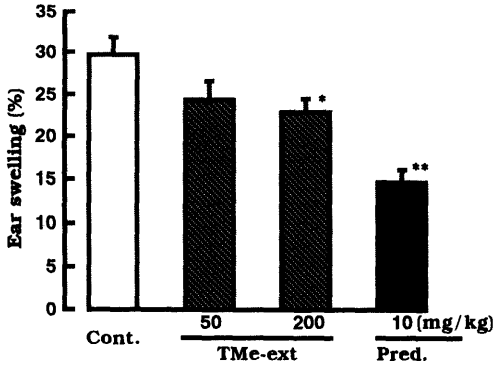

Fig. 6. Effects of Methanol Extract (TMe-ext) from Alismatis Rhizoma and Prednisolone (Pred.) on Induction Phase of Picryl Chloride-induced Contact Dermatitis (PC-CD) in Mice

TMe-ext suspended in 0.5% CMC·Na was orally administered from d −1 to d 6 after sensitization. Prednisolone suspended in 0.5% CMC·Na was orally administered from d 0 to d 6 after sensitization. Control was orally administered 0.5% CMC·Na alone. Ear swelling was measured 24 h after being challenged. Each column represents the mean ± S.E. of 13 mice. Significantly different from the control group, $*p<0.05$, $**p<0.01$.

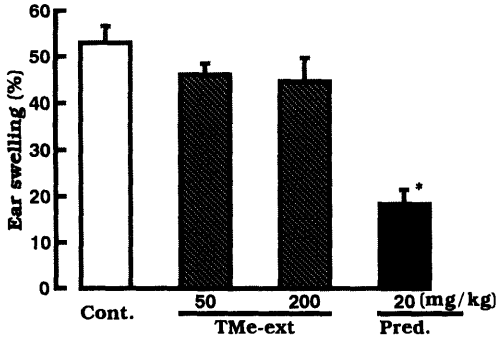

Fig. 7. Effects of Methanol Extract (TMe-ext) from Alismatis Rhizoma and Prednisolone (Pred.) on Effector Phase of Picryl Chloride-induced Contact Dermatitis (PC-CD) in Mice

TMe-ext suspended in 0.5% CMC·Na was orally administered before and 16 h after the challenge. Prednisolone suspended in 0.5% CMC·Na was orally administered 16 h after the challenge. Control was orally administered 0.5% CMC·Na alone. Ear swelling was measured 24 h after the challenge. Each column represents the mean ± S.E. of 13 mice. Significantly different from the control group, $*p<0.01$.

EtOAc-soluble fraction exhibited a superior inhibitory effect compared to that of TMe-ext on DPAR. All six terpenes (Fig. 1) isolated from the EtOAc fraction using silica gel column chromatography also inhibited paw swelling induced by DPAR. Accordingly, it can be considered that these terpenes are active principles in Alismatis Rhizoma concerning anti-type III allergic reactions. Since among these terpenes, alisol B monoacetate was superior to the other five terpenes regarding inhibitory effect, it is presumed to be the most important component contributing to the pharmacological effect of Alismatis Rhizoma.

We previously found that Choreito (a prescription consisting of five crude drugs: Alismatis Rhizoma, Polyporus, Kadinum, Hoelen and Gelatinum) extract (C5-ext) prepared from a decoction of the five mixede drugs, showed an anti-nephritic effect, while the extract (C4+1-ext) prepared from a mixture of the decoction of the four mixed drugs (except for Gelatinum) and the decoction of Gelatinum alone, had no effect.[15] Consequently, it can be considered that in the case of C5-ext, the solubility into the decoction of effective terpenes, which is only slightly soluble in water, was enhanced by decoction together with Gelatinum.[15]

Regarding the pharmacological action of the components in Alismatis Rhizoma, the diuretic and anti-hyperglycemic action of alisol A monoacetate,[16,17] and the diuretic action of alisol B monoacetate[16] and the inhibitory effect of alismol on vascular contraction,[18] have previously been reported, but none of these terpenes seems to be responsible for the anti-type III allergic reaction.

TMe-ext inhibited the IgE-mediated PCA reaction. PCA, an active systemic anaphylaxis, and Schultz-Dale, which are classified as type I allergic reactions, are believed to be caused by chemical mediators such as histamine and leukotrienes being released from mast cells and basophiles by an IgE-related mechanism.

Koda *et al.* reported an increase in the chemical mediator release owing to a water extract of Alismatis Rhizoma on lung slices of sensitized guinea pigs.[19] Accordingly, the effective components in Alismatis Rhizoma on the type I allergic reaction may be similar to lipophilic terpenes which showed an inhibitory effect on the type III allergic reaction.

TMe-ext also significantly inhibited the swelling induced by RCA, one model of type II allergic reactions.

TMe-ext also inhibited an induction phase of PC-CD, but was inactive on the effector phase. Therefore, it can be estimated that TMe-ext indicates an inhibitory effect on type IV allergic reactions by blocking the sensitization for T-cells owing to an antigen.

In view of the inhibitory effects of TMe-ext on the type I to IV allergic reactions, and of six terpenes from TMe-ext on type III allergic reactions, Alismatis Rhizoma can be presumed to be the most important drug in Choreito on experimental nephritis, especially in addition to various allergic reactions.

In conclusion, on clinical application of Alismatis Rhizoma for the treatment of allergic diseases, it is desirable to administer it in preparations of pill or powder from rather in a decoction.

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