Anti-inflammatory Activities of 70% Methanolic Extract from Cinnamomi Cortex

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The effects of 70% methanolic extract (CC-ext) from Cinnamomi Cortex on acute and chronic inflammation were investigated.

CC-ext inhibited the rise in vascular permeability induced by acetic acid and the increase of paw edema induced by carrageenin in mice. It was ineffective on edema derived by histamine or bradykinin, and exhibited only weak inhibitory effect on the edema derived by serotonin. CC-ext also showed inhibitory effects on the prekallikrein enzyme activity and ear edema induced by arachidonic acid. It also had an inhibitory effect on cotton pellet-induced granuloma but showed no atrophying action against the adrenal or thymus glands. Little effect was shown on secondary lesions in the development of adjuvant-induced arthritis (the arthritis reappeared from 11 to 27 d after injection of the adjuvant). These results suggest that some active component having an inhibitory effect on acute inflammation is contained in Cinnamomi Cortex.

Key words Cinnamomi Cortex; *Cinnamomum cassia*; inflammation; carrageenin-induced edema; adjuvant-induced arthritis

Cinnamomi Cortex (the dried cortex of *Cinnamomum cassia*) has been widely used for treatment of headache, fever, hot flushes, cold and physical pain as a stomachic, carminative, corrigent, antipyretic, analgesic and sweating agent in traditional Chinese medicine (Kampo medicine, in Japanese). According to ancient Chinese herbal literature, it is also mentioned as being effective for treatment of Oketsu-syndrome, a pathological concept peculiar to the traditional Chinese system of medicine. Oketsu-syndrome is interpreted to be closely related to disseminated intravascular coagulation (DIC) which is an acquired hemorrhagic disorder characterized by the apparent simultaneous activation of blood coagulation, fibrinolysis and kinin generation combined with the pathologic consequences of fibrin decomposition in the microcirculation.¹⁻³⁾

Oketsu-syndrome is also said to be derived from the advancement of inflammation. Various reports over time have appeared on the pharmacological actions and components of Cinnamomi Cortex, including our previous report that a 70% methanolic extract (CC-ext) from Cinnamomi Cortex exhibited inhibitory effects on blood platelet aggregation induced by collagen, adenosine diphosphate (ADP) and arachidonic acid and on thrombin enzyme activity.⁴⁾ The paucity of reports on the anti-inflammatory effect of Cinnamomi Cortex has now prompted us to investigate this.

MATERIALS AND METHODS

Materials The Cinnamomi Cortex (dried bark of *Cinnamomum cassia* produced in the northern area of Guangdong or Guangxi province, China) given the authors by Nippon Funmatsu Yakuhin, Co., Ltd. (Japan) were used in this study. A voucher specimen is deposited in the Department of Natural Drug Resources, Faculty of Pharmaceutical Sciences, Kinki University. The powdered bark was refluxed at about 85 °C for 2 h (two times) in 70% methanol of decuple of the powder. The

70% methanolic extract was evaporated and then frozen to dryness (CC-ext, yield: 12.8%). The content of its major constituent, cinnamic aldehyde in the extract was determined by high performance liquid chromatography (HPLC) [conditions: column, YMC Pack ODS (A-302) (4.6 mm i.d. × 150 mm); detection, UV absorption at 280 nm; mobile phase, H₂O-MeOH (1:1); flow rate, 0.7 ml/min; column temperature, 40 °C; injection volume, 10 µl; the calibration curve was prepared by an authentic cinnamic aldehyde purchased from Wako Pure Chemicals (Japan)]. The content of cinnamic aldehyde in this extract was 7.7%. The following drugs were also used in this study: λ-carrageenin, serotonin, bradykinin, arachidonic acid, cortisone, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amido (Z-Phe-Arg-MCA) (Sigma), indomethacin, histamine·2HCl, cyproheptadine, soybean trypsin inhibitor (SBTI), kaolin (Nacalai Tesque), diphenhydramine, phenidone, pontamine sky blue (Tokyo Kasei), benzylpenicillin potassium (Meiji Seika), dry heat-killed *Mycobacterium butyricum* (Difco Lab.), Bayol F (Wako Pure Chem.) and gabexate mesilate (FOY, Onoyakuhin Co.).

Animals Male Slc: ddY strain mice (18—20, 30—32 g), male Slc: Wistar strain rats (180—220 g) and female Jcl: SD strain rats (180—220 g) 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, Nihon Nosan Kogyo) and water were given freely.

Acetic Acid-Induced Vascular Permeability in Mice The method was based on that of Whittle.⁵⁾ The ddY strain mice (18—20 g) were dosed orally with the test substances suspended in a 0.2% carboxymethyl cellulose sodium salt (CMC·Na) solution for 1 h before intravenous injection of 4% pontamine sky blue (10 ml/kg). Fifteen min after the injection of the dye, 0.7% acetic acid (10 ml/kg) was injected intraperitoneally. After 20 min, the mice were killed by dislocation of the neck, and the

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viscera were exposed after a 1 min period to allow blood to drain away from the abdominal wall. Each animal was held by a flap of the abdominal wall, and the viscera were irrigated with 10 ml of saline over a petri dish. The washed matter was filtered through glass wool and transferred to a test tube. To each tube was added 0.1 ml of 1 N NaOH in order to clear any turbidity due to protein, and the absorbance was read at 590 nm with a Shimadzu model UV-160 spectrophotometer. Control animals were treated similarly, except that they received an oral dose of the vehicle alone. Vascular permeability was expressed in terms of absorbance value per mouse which leaked into the intraperitoneal cavity. Indomethacin was used as a standard drug.

Carrageenin-Induced Edema in Mice This method was based on that of Nakamura *et al.*⁶⁾ The initial hind paw thickness of the ddY strain mice (18–20 g) was determined by a dial thickness gauge (Mitsutoyo). An 1.5% solution of carrageenin in saline (25 μ l/animal) was injected subcutaneously into the right hind paw 1 h after the test substances suspended in a 0.2% CMC·Na solution had been administered orally. The control group received the vehicle. Paw thickness was measured for up to 6 h at intervals of 1 h, and thickness of the edema was determined. The results were expressed as the percentage of hind paw swelling as compared with the initial hind paw thickness. Indomethacin was used as a standard drug.

Chemical Mediator-Induced Edema in Mice Following determination of initial hind paw thickness of the ddY strain mice (18–20 g), 5 μ l of 1.2% histamine, 0.02% serotonin or 0.6% bradykinin in saline was injected subcutaneously into the right hind paw 1 h after the test substances suspended in a 0.2% CMC·Na solution had been administered orally. The control group received the vehicle. Paw thickness was measured for up to 30 min at intervals of 10 min, and thickness of the edema was recorded. The results were expressed as the percentage of hind paw swelling as compared with the initial hind paw thickness. Diphenhydramine or cyproheptadine was used as a standard drug.

Assay of Prekallikrein Enzyme Activity Whole blood samples were collected from the heart of pentobarbital-anesthetized Wistar strain rats (180–220 g). Nine ml of the blood and 1 ml of sodium citrate (3.8%) were transferred into a plastic tube, and centrifuged at 3000 rpm for 10 min to obtain plasma. Assay of prekallikrein enzyme activity was performed by the method of Ohishi and Katori.⁷⁾ Citrated rat plasma (100 μ l) was mixed with 800 μ l of test solution [2% dimethyl sulfoxide/acetone-Tris·saline buffer (buffer I; 0.02 M Tris·HCl, 0.15 M NaCl, pH 8.0)] and allowed to stand for 10 min at room temperature (about 25 °C). Then, 100 μ l of kaolin suspension (10 mg/ml buffer I) was added and mixed vigorously for 5 s. Five min after the addition of kaolin, 50 μ l aliquots of the reaction mixture were incubated for 10 min at 37 °C with 1 ml of 50 μ M Z-Phe-Arg-MCA in buffer II (0.05 M Tris·HCl, 0.1 M NaCl, 0.01 M CaCl₂, pH 8.0) in the presence (tube A) or the absence (tube B) of 40 μ g SBTI. The reaction was terminated by the addition of 2 ml of 17% acetic acid and the fluorescence was read at 460

(emission) and 380 (excitation) nm in a Hitachi F-4010 fluorescence spectrophotometer. The difference between the value in tube A and B was calculated as prekallikrein activity and the inhibitory percentage of the test substance was determined. FOY was used as a standard drug.

Arachidonic Acid-Induced Ear Swelling in Mice Arachidonic acid-induced ear swelling in mice was performed by the modified method of Young *et al.*⁸⁾ The initial right ear thickness of ddY strain mice (30–32 g) was measured by a dial thickness gauge. One h after the oral administration of test substances suspended in 0.2% CMC·Na solution, each mouse was given 2 mg/ear of arachidonic acid solution (100 mg arachidonic acid/ml acetone) and thickness of the ear was measured 1 h thereafter. The control group received the vehicle. Phenidone used as a standard drug was dissolved in saline and administered intravenously.

Cotton Pellet-Induced Granuloma in Rats The method was based on that of Hicks.⁹⁾ Two cotton pellets (50 $\pm$ 3 mg) were implanted subcutaneously into the back of Wistar rats (180–220 g). The test substances suspended in 0.2% CMC·Na solution were administered once a day for 7 d starting on the day of implantation. The control group received the suspension vehicle and positive control was treated with cortisone (20 mg/kg, *p.o.*). The rats were killed 7 d after the implantation and extraneous tissue was removed from the pellets. Wet weight of the pellets was measured immediately after the removal and the dry weight was measured after drying at 60 °C for 24 h.

Adjuvant-Induced Arthritis in Rats Arthritis was induced by intradermal injection of a 0.05 ml suspension of dry heat-killed *Mycobacterium butyricum* (10 mg) in Bayol F (1 ml) as an adjuvant agent into the tail and right hind paw of SD strain rats (180–220 g). The right hind paw volume of injected adjuvant agent was measured initially, and then every 1–3 d thereafter for 21 d the volume of edema was determined. The results were expressed as the percentage of hind paw swelling as compared with the initial hind paw volume. Thirty days after injection of the adjuvant agent, whole blood samples were withdrawn from the abdominal vein into plastic syringes while the rats were anesthetized with pentobarbital, and euglobulin lysis time (ELT), erythrocyte deformability, platelets, erythrocytes, fibrinogen and fibrin degradation products (FDP) were measured. The ELT was measured by the method of Kaulla and Schultz.¹⁰⁾ Erythrocyte deformability was expressed as erythrocyte filterability measured by the method of Reid *et al.*¹¹⁾ Platelets and erythrocytes were counted with an automatic blood cell counter (Coulter counter, model S-Plus, Coulter Co., U.S.A.). Fibrinogen was determined by the latex aggregation test (FDPL test U, Teikoku Zoki). Test substances suspended in a 0.2% CMC·Na solution were administered orally once a day for 30 d starting on the day the adjuvant was injected. Prednisolone was used as a standard drug.

Statistical Analysis The experimental data were tested for statistically significant differences by the Bonferroni/Dunn's Multiple Range Test.

RESULTS

Acetic Acid-Induced Vascular Permeability The total dye amount which leaked into the peritoneal cavity was 1.17 ± 0.07 OD₅₉₀/mouse in the vehicle control group. When CC-ext (500 mg/kg, *p.o.*) was administered to mice, the dye leakage was inhibited significantly (Fig. 1). A positive control agent, indomethacin 10 mg/kg, *p.o.*, also reduced the leakage.

Carrageenin-Induced Edema CC-ext (500 mg/kg, *p.o.*) had a significant inhibitory effect on the edema 1–6 h after the injection of carrageenin (Fig. 2). Similar inhibition was shown by the standard drug indomethacin (10 mg/kg, *p.o.*).

Chemical Mediator-Induced Edema As shown in Fig. 3, maximal edemas were induced 10 min after injections of the chemical mediators, histamine, serotonin or bradykinin in mice. CC-ext (500 mg/kg, *p.o.*) inhibited the edema induced by serotonin at 20, 30 min after the injection but not that induced by histamine or bradykinin.

Fig. 1. Effects of CC-ext and Indomethacin (Indo.) on Vascular Permeability Induced by Acetic Acid in Mice

CC-ext and indomethacin suspended in 0.2% CMC·Na were administered orally 1 h before the intravenous injection of 4% pontamine sky blue dissolved in saline (10 ml/kg). Fifteen min after injection of the pontamine sky blue, 0.7% acetic acid was injected intraperitoneally (10 ml/kg). After 20 min the mice were killed and the vascular permeability was measured and expressed in terms of OD₅₉₀ as amount of dye which leaked into the intraperitoneal cavity. Each column represents the mean $\pm$ S.E. of 15–17 mice. Significantly different from the control group, **p* < 0.05, ***p* < 0.01.

tion but not that induced by histamine or bradykinin. Positive control agents, diphenhydramine and cyproheptadine exhibited an inhibitory effect against the histamine, bradykinin-induced edema and serotonin-induced edema, respectively.

Prekallikrein Enzyme Activity As shown in Fig. 4, CC-ext at concentrations of 100–500 μ g/ml inhibited the enzyme activity in a dose dependent manner. A positive control agent, FOY (50, 100 μ g/ml) also showed significant inhibition.

Arachidonic Acid-Induced Ear Swelling CC-ext (200 mg/kg, *p.o.*) had a significant effect on the ear swelling induced by arachidonic acid (Fig. 5). A positive control agent, phenidone (20 mg/kg, *i.v.*) also showed the inhibition.

Cotton Pellet-Induced Granuloma As shown in Table 1, cortisone (20 mg/kg, *p.o.*) was effective in reducing wet and dry weight of granuloma and exhibited an atrophying effect on adrenal gland and thymus. CC-ext (500 mg/kg, *p.o.*) also showed the inhibitory effect but not the atro-

Fig. 2. Effects of CC-ext and Indomethacin on Carrageenin-Induced Paw Edema in Mice

An edema on the right foot of mice was induced by subcutaneous injection of 1.5% λ -carrageenin (25 μ l/animal) dissolved in saline. One h prior to this, each test substance suspended in 0.2% CMC·Na was administered orally. Control was orally administered 0.2% CMC·Na alone. Each point represents the mean $\pm$ S.E. of 9–10 mice. Significantly different from the control group, **p* < 0.05, ***p* < 0.01. ○, control; ▲, CC-ext 50 mg/kg; ■, CC-ext 200 mg/kg; ▼, CC-ext 500 mg/kg; ●, indomethacin 10 mg/kg.

Fig. 3. Effects of CC-ext, Diphenhydramine or Cyproheptadine on Histamine, Serotonin or Bradykinin-Induced Paw Edema in Mice

An edema on the right foot of mice was induced by subcutaneous injection of 1.2% histamine, 0.02% serotonin or 0.6% bradykinin dissolved in saline (5 μ l/animal). One h prior to this, each test substance suspended in 0.2% CMC·Na was administered orally. Control was administered 0.2% CMC·Na alone. Each point represents the mean $\pm$ S.E. of 9–10 mice. Significantly different from the control group, **p* < 0.05, ***p* < 0.01. ○, control; ▲, CC-ext 50 mg/kg; ■, CC-ext 200 mg/kg; ▼, CC-ext 500 mg/kg; ●, diphenhydramine 50 mg/kg; □, cyproheptadine 2 mg/kg.

Fig. 4. Effects of CC-ext and FOY on Prekallikrein Activity of Rat Plasma

One hundred μ l of rat plasma was mixed with test substance and allowed to stand for 10 min at room temperature. Five min after the addition of 1.0% kaolin suspension, 50 μ l aliquots were taken and incubated for 10 min with Z-Phe-Arg-MCA in the presence or the absence of the SBTI. The reaction was terminated by the addition of 17% acetic acid and the fluorescence was read. The difference of fluorescence was expressed as prekallikrein activity. Each column represents the mean $\pm$ S.E. of 3 experiments. Significantly different from control, * p < 0.01.

Fig. 5. Effects of CC-ext and Phenidone (Phen.) on Arachidonic Acid-Induced Ear Edema in Mice

The ear edema of mice was induced by application of 2 mg arachidonic acid dissolved in acetone (20 μ l/animal). One h after the application of arachidonic acid, ear thickness was measured and expressed as ear swelling compared with the thickness before the application. Each test substance suspended in 0.2% CMC·Na was administered orally 1 h before the application. Controls were orally administered 0.2% CMC·Na or intravenously injected with saline alone. Each column represents the mean $\pm$ S.E. of 8–10 mice. Significantly different from the control group, * p < 0.01.

Table 1. Effects of CC-ext and Cortisone on Wet and Dry Weights of Cotton Pellet Granuloma in Rats

Treatment	Dose (mg/kg)	Weight of granuloma (mg)		Ratio to body (mg/100 g)	
		Wet	Dry	Thymus	Adrenal glands
Control	—	894 $\pm$ 35	160 $\pm$ 8.5	227 $\pm$ 9.7	17.06 $\pm$ 0.56
CC-ext	50	818 $\pm$ 24	139 $\pm$ 4.7*	231 $\pm$ 8.6	16.98 $\pm$ 0.39
	200	825 $\pm$ 35	139 $\pm$ 7.8*	233 $\pm$ 9.9	17.28 $\pm$ 0.57
	500	780 $\pm$ 26*	133 $\pm$ 5.6**	214 $\pm$ 9.3	17.96 $\pm$ 0.65
Cortisone	20	774 $\pm$ 38*	132 $\pm$ 7.2**	203 $\pm$ 8.1*	16.71 $\pm$ 0.56

Cotton pellet granulomas were induced in rats by implantation of cotton weighing about 50 mg in the animal's back. Each test substance suspended in 0.2% CMC·Na was administered orally for 7 d, once/d from 0 to 6 d after the operation. Cotton pellet granulomas were removed on the 7 d, and wet and dry weights of granulomas were measured. Control was orally administered 0.2% CMC·Na alone. Each value represents the mean $\pm$ S.E. of 8–10 rats. Significantly different from the control group, * p < 0.05, ** p < 0.01.

Fig. 6. Effects of CC-ext and Prednisolone on Right Hind Paw Edema of Adjuvant-Induced Arthritis in Rats

Arthritis was induced by intradermal injection of a 0.05 ml suspension of dry heat-killed *Mycobacterium butyricum* (10 mg) in Bayol F (1 ml) as adjuvant agent into tail and right hind paw of rats. Each test substance suspended in 0.2% CMC·Na was orally administered once/d for 21 d immediately after the injection of adjuvant agent. Each point represents the mean $\pm$ S.E. of 11–13 rats. Significantly different from the control group, * p < 0.05, ** p < 0.01. ○, control; ▲, CC-ext 50 mg/kg; ■, CC-ext 200 mg/kg; ▼, CC-ext 500 mg/kg; ●, prednisolone 10 mg/kg.

phying effect.

Adjuvant-Induced Arthritis As shown in Fig. 6, CC-ext (500 mg/kg, *p.o.*) had an inhibitory effect on the

development of edema 13–15 d after the injection of adjuvant in adjuvant-induced arthritic rats. The standard drug, prednisolone (10 mg/kg, *p.o.*) showed a strong inhibition of the edema. Further, 30 d after the injection of adjuvant, various hemorheological parameters were measured. The fibrinogen and FDP contents were increased and platelet counts tended to increase more in the arthritic rats than in the normal rats (data not shown). The fibrinolytic activity and the erythrocyte deformability were also reduced. No preventive effect of CC-ext was recognized on the changes in blood rheology.

DISCUSSION

The anti-inflammatory effects of CC-ext were investigated using various experimental models. CC-ext (50 to 500 mg/kg, *p.o.*) dose-dependently exhibited an inhibitory effect on the increased vascular permeability induced by acetic acid in mice, the typical model of the first stage of inflammatory reaction.

To determine the inhibitory effect against carrageenin-induced edema which was modelled in the first and second stages of inflammatory reaction, experiment was done

using mice in place of rat in order to minimize the total quantity of testing sample which is consumed in experiment. Because a relatively large quantity of the sample will be required to pursue an active principle in CC-ext by monitoring anti-inflammatory activity for future study. The edema formed in mice was slightly different from that in rat in overall aspect and the formative process of swelling. Nevertheless, the effect of CC-ext on the acute inflammation induced by carrageenin can also be estimated even in mice, since indomethacin, a typical anti-inflammatory agent, inhibited edema induced by carrageenin in these animals.

In practice, CC-ext showed an inhibitory effect on carrageenin-induced edema when given orally to mice at the dosage of 50 to 500 mg/kg. This effect was relatively strong on the increased edema 3 h after injection, but weak 1 h after the injection. The biochemical mechanism for inflammatory reaction in the organism (the carrageenin edema) is not yet clear because various factors are involved in the induction of inflammation. However, chemical mediators such as histamine, serotonin, prostaglandin (PGs) and kinin are presumed to play an important part in the occurrence and development of inflammation. Edema induced by carrageenin is divided into the first phase with the participation of histamine or serotonin and the second phase in which PGs or bradykinin participate through the advance and retreat of swelling.

CC-ext was ineffective on edema derived from histamine or bradykinin and showed only weak inhibition on that derived from serotonin. Accordingly, it is believed to have only a slight inhibitory effect against chemical mediators related to the first phase of carrageenin-induced edema. Since CC-ext did, in fact, inhibit the second phase of carrageenin-induced edema, there is no gainsaying the possibility that it possesses some effect on the formation or elimination of kinins and PGs in the edema.

The effect of CC-ext on the activation of prekallikrein (an important enzyme in the process of kinin formation) and on arachidonic acid cascade was then studied. CC-ext showed an inhibitory effect on the activation of kallikrein but not on bradykinin-induced edema. It also significantly inhibited an ear edema induced by arachidonic acid. The inhibitory mechanism of CC-ext on carrageenin-induced edema is thought to account for the resulting inhibitory actions against kinin formation and arachidonic acid cascade.

CC-ext inhibited cotton pellet-induced granuloma, but showed no atrophying action against the adrenal or thymus glands. Cortisone inhibited cotton pellet-induced granuloma and decreased the weight of both glands. Accordingly, it is obvious that the mode of action of CC-ext is different from that of steroids.

The extract showed a weak inhibitory effect on the secondary lesions in developing adjuvant-induced arthritis (the arthritis reappeared 11 to 27 d after injection of the adjuvant). The secondary lesion is thought to be related to the formation of antibody or activation of complement, and may involve type III or IV allergic reaction.^{12,13} The weak inhibitory effect of CC-ext on adjuvant-induced

arthritis is believed attributable to its inhibition of the type IV allergic reaction and complement activation.

We have reported that the animal model in adjuvant-induced arthritis resulted in the change of hemorheology caused by increased coagulation and decreased fibrinolysis, as well as a multiplication of connective tissue. This is believed to be in accordance with the pathologic view of Oketsu syndrome of the traditional Chinese system of medicine (Kampo medicine, in Japanese). Kampo prescriptions (Keishi-bukuryo-gan, Daio-shachu-gan and others) or crude drugs (Jio, Wogon *etc.*) which have been used for treatment of Oketsu syndrome, were previously found to inhibit the change of hemorheology induced in the animal model of adjuvant-induced arthritis.¹⁴⁻¹⁶

However, CC-ext showed no effect on the lowering of fibrinolytic action or of transformable function of erythrocytes which are responsible for microcirculation disorder in adjuvant-induced arthritis; nor did it affect the blood parameters such as the number of erythrocytes or the increase of fibrinogen and FDP.

In this report, it has become apparent that CC-ext has inhibitory activity against inflammation in the relatively acute stage. It showed little effect on adjuvant-induced arthritis and was also ineffective in varying blood parameters in the chronic inflammatory model. Therefore, the effect of Cinnamomi Cortex on the Oketsu syndrome, the symptom name used in Kampo medicine, is dependent on the direct inhibitory action against any abnormal behavior derived from an inflammation interpreted to be a factor of this syndrome, without improving the microcirculation disorder caused by chronic immunodeficiency disease. Further study is in progress to clarify the active principles in CC-ext on acute inflammation and the mechanism of its action.

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