Nutraceuticals & Food
Vol. 7, p.347~352 (2002)

Nutraceuticals and Food

Anti-rheumatoidal Effect of Sulfuretin Isolated from the Heartwood of *Rhus veniciflua* in Rats and Mice

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Abstract

The present study was undertaken to evaluate the anti-rheumatoidal arthritis effect of the *R. verniciflua* heartwood extract, its EtOAc fraction, and its primary flavonoids, sulfuretin and fustin. All test samples showed variably significant inhibitory effects on hind paw edema and trypsin inhibitor activity induced by Freund's complete adjuvant reagent (FCA reagent), and on vascular permeability caused by acetic acid. Treatment with 10 mg/kg (i.p.) sulfuretin for seven days inhibited edema formation by 54.2±3.0%. Test samples, especially sulfuretin, shifted the values of biochemical parameters such as serum-cholesterol, serum-triglyceride and serum-total protein toward the normal and restored the numbers of leucocytes and platelets. These results suggest that the heartwood of *R. verniciflua* reduces immunological injuries caused by FCA reagent provides evidence that suluretin is an active anti-rheumatoid arthritis agent.

Key words: *Rhus verniciflua*, sulfuretin, anti-rheumatoid arthritis

INTRODUCTION

The stem bark or its exudate of *Rhus verniciflua* (Anacardiaceae) have been used to treat a variety of aging-related diseases (1). However, urushiols of this plant form polymers, though the laccase action of the plant, that can cause severe allergic reactions (2). Although many patients use the barks or exudates of this plant for the medicinal purposes, many atopic people must avoid its use because of the severity of the allergic reactions. In contrast, the heartwood of this plant does not evoke allergic reaction, implying that the heartwood contains neither urushiols nor laccase.

Rhus veniiciflua is used in Korean folkloric medicine as a beverage ingredient, crude drug and traditional tea for the treatment of lingering intoxication, heavy smoking and inflammatory diseases such as rheumatoid arthitis. In our efforts to characterize the anti-rheumatoidal action of the *R. verniciflua* heartwood, we attempted to isolate the components responsible for the anti-rheumatoidal effects in rats with rheumatoid arthritis. Therefore, we report the effects of the heartwood extracts and the active principle, sulfuretin.

MATERIALS AND METHODS

Plant material and preparation of test samples

The heartwoods of *Rhus verniciflua* stokes grown on Chiak Mountain in the Kangwon Province, Korea were collected in September 1999, and the identity confirmed by Prof. G. T. Kim (Division of Applied Plant Sciences, Sangji University, Wonju, Korea). A voucher specimen (# natchem-18) was deposited in the herbarium at the Department of Applied Plant Sciences, Sangji University, Wonju, Korea.

Dried heartwoods (2 kg) of *R. verniciflua* were cut and extracted three times with MeOH under reflux and evaporated to give a viscous mass (280 g). This material was suspended in 3 L H_2O and then consecutively partitioned with 3 L each of $CHCl_3$, EtOAc and *n*-BuOH. Each layer was dried *in vacuo* to yield $CHCl_3$ (68 g), EtOAc (95 g), and *n*-BuOH (70 g) fractions. A part of the EtOAc fraction (20 g) was chromatographed over silica gel (600 g, 770 cm; Art 7734, Merck, Germany) with eluting solvent of $CHCl_3$-MeOH-H_2O (73:27:10, lower phase, 5 L) to give 5 fractions (fraction 1~5) for further isolation. Eluents of 80 mL each were collected to afford 70 frac-

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tions, each of which was checked under UV light (254 and 365 nm) or 50%-H_2SO_4. The fractions showing similar patterns on TLC were grouped together and evaporated on a rotary evaporator to give 5 fractions (fractions 1~5). Repeated column chromatography of fraction 3 (1,040~1,200 mL) were carried out over silica gel with eluting solvent of $CHCl_3$-MeOH (10:1) and yielded orange-yellow needles (**1**, Rf 0.65, 74 mg) after recrystallization from the MeOH solution. Fraction 5 (1,360~1,600 mL), which still contained impurities, was subjected to ODS and Sephadex LH-20 column chromatography to yield compound **2** (Rf 0.53, 190 mg). Compounds **1** and **2** were identified as sulfuretin and fustin, respectively, by physicochemical and spectroscopic analysis. {**1**: Orange-yellow prisms (160 mg) from MeOH, mp 280~285 (dec.); EI-MS (70 eV) m/z: 270.3 (M^+, $[C_{15}H_{10}O_5]^+$) (3); **2**: White needles (250 mg) from MeOH, mp 228~229, $[\alpha]_D$+28.3(c, 0.9 in 50% aqueous acetone); EI-MS (70 eV) m/z: 288.3 (M^+, $[C_{15}H_{12}O_6]^+$) (4)}. Purified sulfuretin and fustin were used for testing in the animal experiments, as well as the non-purified MeOH extract and EtOAc fraction.

Preparation of test sample solutions

Test samples (MeOH extract, EtOAc fraction, sulfuretin, fustin and prednisolone) were first dissolved in 10% tween 80 and then diluted in saline solution. The same volume of solvent alone was administered to the normal group. The extract and fractions were orally administered at dosages of 150 and 250 mg/kg, respectively, and the isolated compounds (sulfuretin and fustin) were intraperitoneally administered at 5 and 10 mg/kg, respectively, based on observations made in preliminary experiments.

Animals

Both four week-old Sprague-Dawley male rats and ICR male mice were purchased from Korean Experimental Animal Co. and allowed to adapt for at least two weeks before beginning the experiment. All animals were kept in a controlled environment at a temperature of 20±2°C, 40~60% relative humidity, and a 12 hr light/dark cycle. The animals were only given water for twenty-four hours prior to beginning the experiment. Considering the variation of enzyme activity throughout the day, the animals were sacrificed at a fixed time (10:00 AM~12:00 AM).

Induction of rheumatoid arthritis

Each 0.05 mL of Fruend's complete adjuvant reagent (FCA reagent; Difco) was injected into the right footpads of the rats. Two weeks later, the induction of rheumatoid arthritis in rats was observed. The test samples were dissolved in DMSO and diluted to various concentrations with saline. The test solutions {each, 150 and 250 mg/kg (p.o.); 5 and 10 mg/kg (i.p.)} were administered for 3, 5, 7, 10 days. The effect was taken by plethysmometer (Ugo Basile, Italy). The inhibitory effect was calculated as follows: Inhibitory effect of edema (%)=(volume of control group-volume of treatment group/volume of control group)×100. After the final treatment with the test samples, the animals were anesthetized and blood was collected after decapitation from the abdominal aorta. The free flowing blood was preserved in CBC bottles, and the remnants coagulated at room temperature by standing for 30 min. The serum was collected by centrifugation (600 g, 15 min).

Activity of trypsin inhibitor

The trypsin inhibitor activity was measured according to the methods of Bieth et al. (5) and Fritz et al. (6). The absorbance of N-benzoylarginine-p-nitroanilide (BAPNA) was measured at 405 nm, which measures the conversion of BAPNA to *N*-benzoylarginine and *p*-nitroaniline by trypsin activity. In brief, trypsin solution (50 mU/mL) and triethanolamine buffer solution (0.2 M triethanolamine, 20 mM $CaCl_2$, pH 7.8) were added to the test tubes prepared for the inhibitor assay (I) and trypsin reference assay (Tr), respectively; and pre-incubated at 37°C for 5 min. Next, the reaction was initiated by adding the BAPNA substrate. The reaction rate was determined every 1 min for 5 min by the absorbances taken at 405 nm by a UV-VIS spectrophotometer. Inhibitor units (IU) was determined by the following formula: inhibitor activity (IU)=(ΔA_{Tr}/min - A_I/min)/3.32, where A represents the absorbance. The unit of enzyme activity was expressed as IU/mL.

Vascular permeability test

Thirty min after the injection (i.p., 0.7% acetic acid-saline 0.1 mL/10 g), 4% pontamine was also injected to tail vein (7). Twenty min after the injection of the pigment, the mouse was sacrificed and then the pigment exudated into the abdominal cavity was washed with 10 mL of distilled water. This washed solution was centrifuged (3,000 rpm, 10 min) and then the absorbances of supernatants were measured at 580 nm wavelength using a UV-Vis spectrophotometer. Test solutions were orally or intraperitoneally administered 30 min before the injection of acetic acid-saline solutions.

Determination of LDH activity in blood

For determination of lactate dehydrogenase (LDH) activity in FCA reagent-treated rats, the lactate substrate method (8) was used. This experiment produces diformazine the reduced form of nitrotetrazolium blue under 1-methoxy-5-ethylphenazynium methylsulfate by NADH formed in the dehydrogenation process from lactate to pyruvate.

Determination of serum triglyceride and total cholesterol

Total serum cholesterol concentrations were enzymatically

Table 1. Time and dose responses of the extracts and components of *Rhus veniciflua* on anti-inflammatory effect in rats induced by Freund's complete adjuvant reagent

Group	Dose (mg/kg)	Swelling percent				
		0	3	5	7	10 (day)
Control	-	75.6±4.3	79.7±3.2	78.4±3.2	81.3±4.4	84.2±3.6
MeOH ext.	150		74.4±3.7*	75.2±2.4	72.8±3.3*	73.4±2.5*
	250		72.9±3.2*	73.8±2.2	70.5±5.1*	70.2±2.1*
EtOAc ext.	150		70.8±3.4*	71.4±4.2*	69.8±2.4*	68.5±3.0*
	250		69.3±3.2*	68.5±2.2*	67.3±3.2*	63.9±2.4*
Sulfuretin	5		68.8±2.0*	67.8±2.0*	65.3±2.1*	63.4±3.0**
	10		60.9±2.1**	59.3±2.2**	55.6±3.2**	54.2±2.2**
Fustin	5		72.8±3.2*	71.8±4.0*	70.4±3.2*	69.7±4.0*
	10		65.4±2.4*	67.9±3.1*	63.5±1.9*	62.5±2.4*
Prednisolone	10		37.6±3.1***	25.3±2.7***	20.9±2.5***	15.6±3.2***

Rats were orally and intraperitoneally administered *R. veniciflua* daily for seven days. Values represent means±SE (n=10). *p<0.05, **p<0.01, ***p<0.001 compared with the control as analyzed with the Student's t-test.

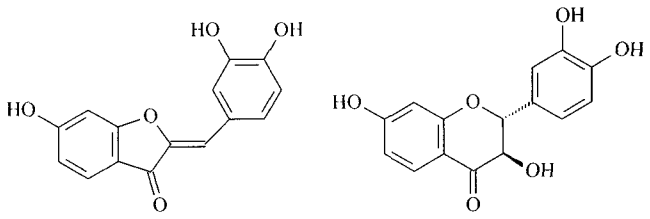
determined using a commercial kit (9). The enzyme solution 3.0 mL was added to 200 mL of the serum and mixed. After incubation at 37°C for 5 min the absorbance was measured at 500 nm. Serum triglyceride concentrations were determined using a commerical kit according to McGowan's method (10).

Measurement of erythrocytes, leucocytes, hemoglobin, hematocrit and platelets, and determination of total protein

Effects on erythrocytes, leucocytes, hemoglobin, hematocrit and platelets were measured using a Sysmex K-1000 Cell-Counter according to Fonio's method (11). The content of total protein was determined using a commercial kit based on the Biuret method (12).

RESULTS

Compounds **1** and **2** were identified as sulfuretin and fustin, respectively, by physicochemical and spectroscopic analysis (mp, $[\alpha]_D$, EI-MS) (Fig. 1). Identities of compounds were confirmed by comparison with known standards on TLC. All the treated groups showed significant anti-edema effects but were less effective than prednisolone-treated group (Table 1). Higher doses or longer treatment durations resulted in greater decreases in the swelling rate of the hind paws. The activity of the EtOAc fraction was more potent than that of the MeOH extract, suggesting that the most active components exist in the EtOAc fraction. The potency of sulfuretin was stronger than fustin. The trypsin inhibitor activity of 0.179 IU/mL in the normal group was considerably increased to 0.277 IU/mL by FCA reagent administration (Table 2). Prednisolone treatment potently decreased the trypsin inhibitor activity, as did the sample treatments which exhibited a variably significant inhibition. Treatment with 5 mg/kg sulfuretin showed very weak inhibition, but when it was increased to 10 mg/kg provided much more significant inhibitory rate.



Sulfuretin Fustin

Fig. 1. Structures of sulfuretin and fustin isolated from the heartwood of *R. verniciflua*.

Table 2. Inhibitory activity of the extracts and components of *R. verniciflua* on the hydrolysis BAPNA by trypsin

Group	Dose (mg/kg)	Activity IU/mL	% Inhibition rate
Normal	-	0.179±0.018	0
Control	-	0.277±0.029	2.0
MeOH ext.	150 (p.o.)	0.275±0.017	17.3
	250 (p.o.)	0.260±0.015	14.3
EtOAc ext.	150 (p.o.)	0.263±0.021	24.5
	250 (p.o.)	0.253±0.020	12.2
Sulfuretin	5 (i.p.)	0.265±0.015	12.2
	10 (i.p.)	0.217±0.018*	61.2
Fustin	5 (i.p.)	0.270±0.013	12.2
	10 (i.p.)	0.236±0.014*	41.8
Prednisolone	100 (p.o.)	0.181±0.013**	98.0

Ten rats were orally and intraperitoneally administered with the extracts or components of *R. verniciflua* daily for seven days. Values represent means±SE (n=10).
*p<0.05, **p<0.01 compared with the control as analyzed with the Student's t-test.

Since the edema is associated with vascular permeability, we measured the dye leakage into the peritoneal cavity in mice induced by acetic acid. The active flavonoid, sulfuretin, significantly reduced the dye leakage (Table 3). Therefore, the anti-rheumatoidal effect of the heartwood of *R. vernificiflua* was confirmed by observation of the inhibitory effects on edema, trypsin inhibitor and vas-

Table 3. Effect of the extracts and components of *R. verniciflua* on the dye leakage into the peritoneal cavity induced by acetic acid in mice

Group	Dose (mg/kg)	Permeability dye amount (μg)	Inhibition rate (%)
Normal	-	103.6±12.7	-
Control	-	243.2±11.2	100.0
MeOH ext.	150 (p.o.)	234.7±10.7	6.0
	250 (p.o.)	201.2± 6.5*	30.1
EtOAc ext.	150 (p.o.)	240.9±13.7	1.6
	250 (p.o.)	190.5±10.8*	37.8
Sulfuretin	5 (i.p.)	210.2±10.2*	23.6
	10 (i.p.)	172.9± 9.3**	50.3
Fustin	5 (i.p.)	220.8± 8.5*	16.0
	10 (i.p.)	193.5±12.2*	35.6
Prednisolone	100 (p.o.)	107.8±11.6***	97.0

Ten mice were orally and intraperitoneally administered the extracts or components of *R. verniciflua* daily for seven days. Values represent means±SE (n=10).
*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the control as analyzed with the Student's t-test.

Table 4. Effect of the extracts and components of the *Rhus verniciflua* heartwood on the hematological values in rats treated with FCA reagent

Group	Dose (mg/kg)	Leucocyte (10^3/μL)	Platelet (10^3/μL)
Normal	-	13.8±2.5	200.4±13.9
Control	-	31.7±3.1	227.6±12.4
MeOH ext.	150	29.0±2.6	224.2±13.6
	250	26.2±3.2*	210.5±14.5
EtOAc ext.	150	28.7±2.4	230.9±13.6
	250	25.2±2.5*	211.3±14.3
Sulfuretin	5	28.5±2.5	200.4±12.5*
	10	20.4±2.5**	190.2±14.1*
Fustin	5	29.1±2.4	193.9±11.3*
	10	23.6±2.6*	200.7±13.6*
Aminopyrine	100	11.9±2.0***	180.5±13.1**

Values represent means±SE (n=10).
*$p<0.05$, **$p<0.01$, $p<0.001$ compared with the control as analyzed with the Student's t-test.

cular permeability.

We successively observed hematological changes such as erythrocyte, leucocyte and platelet counts; and hemoglobin and hematocrit in rats with induced rheumatoid arthritis (Table 4). FCA reagent treatment considerably increased leucocytes in blood from $(13.8\pm2.5)\times10^3$/μL in normal rats, and to $(31.7\pm3.1)\times10^3$/μL in control rats. Platelet counts also increased signifiacantly. However, there were no significant changes in erythrocytes, hemoglobin or hematocrit, and, likewise, none of the treatment groups showed any marked changes (data not shown). Every treatment significantly prohibited those hematological changes.

FCA reagent administration considerably increased serum concentrations of triglyceride, total protein and LDH, and significantly decreased total cholesterol (Table 5). Prednisolone treatment potently shifted the values toward those of normal animals. The active component, sulfretin, exhibited significant activities in the hematological parameters as shown in Table 5.

DISCUSSION

FCA reagent is sterilized *Mycobacterium tuberculosis*, and surfactant-added fluid paraffine (13,14). FCA reagent, in general, induces chronic inflammation in two weeks in contrast to the induction of acute inflammation by carrageenan administration. Rheumatoid arthritis is a disease caused by a series of auto-immune reactions which result in inflammation and tissue damage (15). A characteristic feature of rheumatoid disease is the increase of reactive oxygen species that can be observed in organs related with the site of inflammatory response. Inhibitory effects of test substances on edema and trypsin inhibitor

Table 5. Effect of the extracts and components of the *Rhus verniciflua* heartwood on serum triglyceride, total cholesterol, total protein and LDH in rats treated with FCA reagent

Treatment	Dose (mg/kg)	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	Total protein (g/dL)	LDH (U/L)[1)]
Normal	-	70.8±3.1	77.8±2.2	6.18±1.33	721.2±48.2
Control	-	125.2±4.1	51.6±2.0	9.42±1.27	1321.8±87.5
MeOH ext.	150	110.6±2.6	52.4±3.2	9.30±1.40	1011.3±53.7
	250	107.2±2.1*	58.9±2.3*	8.87±2.70	938.8±43.2*
EtOAc ext.	150	105.5±3.1*	54.2±3.1	9.16±1.54	993.7±29.4
	250	93.2±5.2*	60.4±3.0*	8.63±2.36	903.5±28.5*
Sulfuretin	5	102.7±2.5*	54.3±2.2	9.03±2.00	924.2±18.6*
	10	87.4±3.2**	65.7±4.1**	8.10±1.92*	831.4±20.2**
Fustin	5	108.8±4.2*	58.6±1.4*	9.14±1.55	995.8±34.6
	10	99.2±3.5**	62.9±3.4*	8.54±1.77*	864.9±30.3*
Prednisolone	100	74.6±4.2**	73.4±2.0**	6.43±1.34**	753.6±15.5**

[1)]Wroblewski unit.
Values represent means±SE (n=10).
*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the control as analyzed with the Student's t-test.

ctivity, induced by FCA reagent, and on vascular permebility, induced by acetic acid, demonstrated that they re efficacious treatments for rheumatoid arthritis. The igher activities of EtOAc extract than MeOH extract uggest that the same flavonoids distributed in EtOAc raction might be responsible for the activity of the MeOH xtract. From the EtOAc extract, two major flavonoid comonents, sulfuretin and fustin, were isolated. Although oth sulfuretin and fustin were bioactive, sulfuretin had he most significant effect in the anti-edema test. Therefore, ulfuretin, an aurone-type flavonoid, was the most potent lavonoid in anti-rheumatoid arthritis activity tests. Treatnent of 10 mg/kg sulfuretin suppressed the rheumatoid rthritis much more potently than 5 mg/kg treatment. rednisolone, a steroidal anti-inflammatory drug, has an mmunosuppressive action and, therefore, it can inhibit heumatoid arthritis caused by auto-immune reactions. Ve previously reported the significant anti-rheumatoid rthtitis effects of kalopanaxsaponin A isolated from *Kalopanax pictus* (16-18). Based on the similar feature f sulfuretin with kalopanaxsaponin A on rats with rheunatoid arthritis, precise biochemical and pharmacological esearch is needed for the elucidation of the mechanism f action. We now presume that the immunosuppressive eature of sulfuretin may contribute to the anti-rheumaoid arthritis effect as with prednisolone.

Since rheumatoid arthritis is a systemic, rather than ocal, disease, the syndromes can also be observed from nany of biochemical parameters. Considerable increases n leucocyte counts following FCA reagent treatment nay be a diagnosis of rheumatoidal diseases since the eagent contains sterilized *Mycobacterium tuberculosis*. n addition, slight increase in platelet counts were also bserved, and test substances reduced these increases. n general, leucocyte counts are increased during diseases uch as infection, inflammation, tissue necrosis, hemolysis, nalignant tumors and leukemia. Increased rates of lipid eroxidation cause changes in serum lipids and serum roteins, as well as serum LDH activity. To investigate he effects of sulfuretin, fustin, and the EtOAc extract on ipid metabolism in FCA-treated rats, serum triglyceride nd cholesterol concentrations were measured. High trigyceride concentratons are typically found in patients with liseases of atherosclerosis, hyperlipemia, and gout (19). riglyceride concentrations in the sulfuretin treatment roup (10 mg/kg) were lowered to that of a normal group. n FCA-treated rats, the triglyceride lowering effect of ulfuretin was very strong. This is, to some extent, in greement with reports indicating that there are high viscosities of both total blood and serum in patients with heumatoidal arthritis. Lower than normal levels of cholesterol are found in patients with hypertyroidism, malignant tumors, inflammatory diseases, diffuse collagen disease and anemia (19). The considerable changes in biochemical parameters could reflect the involvement of reactive oxygen species in rheumatoid arthritis. The increases in serum LDH activity, may indicate that the FCA reagent causes liver damage. All the indicators suggested that a systemic disorder was induced by the FCA reagent. Therefore, our results suggest that sulfuretin prevented the pathological progression of the immunological reaction. Furthermore, the flavonoid rich extracts of *Rhus veniciflua* extracts may be effective in preventing or treating rheumatoid arthritis.

ACKNOWLEDGEMENTS

This research was supported by a grant (PF002104-07) from Plant Diversity Research Center of 21st Frontier Research Program funded by Ministry of Science and Technology of Korean Government.

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(Received September 10, 2002; Accepted November 26, 2002)