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A COMPARATIVE STUDY ON ANTI-INFLAMMATORY ACTIVITIES OF THE ENANTIOMERS, SHIKONIN AND ALKANNIN

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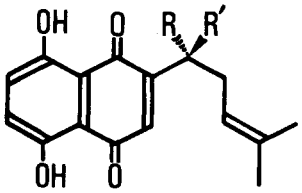
ABSTRACT.—A pharmacological comparison between the enantiomers, shikonin (*R*) and alkannin (*S*), was made with regard to their inhibitory effects on the increased capillary permeability and thermal edema in rats, using phenylbutazone as a positive control in both models. The results of experiments have shown that there is no significant difference in the anti-inflammatory activity between the two compounds.

Ointments prepared from the purple roots of the two boraginaceous plants *Lithospermum erythrorhizon* Sieb. et Zucc. and *Alkanna tinctoria* Tausch have been used for burns, inflammations, wounds, and ulcers since ancient times in the Far East and Europe, respectively (1). The root of the former species contains red naphthoquinone pigments, shikonin derivatives (*R*-configuration) (2,3), whereas that of the latter accumulates the corresponding enantiomers, alkannin derivatives (*S*-configuration) (3). Shikonin shows antibacterial (4,5), anti-inflammatory (6), antitumor (7), and wound-healing activities (6), while alkannin has been reported to be an effective cure for chronic skin ulcer by clinical tests (1,8). However, no direct comparison between shikonin and alkannin has yet been made concerning the pharmacological activities under the same experimental conditions.

The objective of the present study was to compare the anti-inflammatory activities of these optical isomers, which were externally applied as ointments to the test animals.



Shikonin (R=OH, R′=H)
Alkannin (R=H, R′=OH)

RESULTS AND DISCUSSION

Shikonin (*R*) and alkannin (*S*) are mutually distinguished by the configuration of the hydroxy group at C-1 of the isohexenyl side chain, and an equimolar mixture of these enantiomers is called shikalkin (3, 9).

It has been reported by Hayashi (6) that the topical application of shikonin (50 μg) caused a 40% inhibition of the increased vascular permeability induced by histamine. In the present experiment, the topical application of either alkannin or shikonin (50 or 100 μg) to the rats equally tended to decrease the increased capillary permeability induced by an intradermal injection of histamine (Table 1). It was ascertained by the control experiment that the ointment base without the pigment was pharmacologically inactive.

Table 2 shows the anti-inflammatory effects of shikonin, shikalkin, and alkannin on the edema caused by a thermal injury to the skin of rats. Shikonin, shikalkin, and alkannin were found to be equally effective in inhibiting the thermal edema at two dos-

TABLE 1. Effects of Shikonin and Alkannin on Increased Capillary Permeability after Intradermal Injection of Histamine in Rats

Test compound	Topical dosage (μg)	Dye leakage (μg)		Inhibition (%)
		Control	Treatment	
Shikonin	50	24.9±3.1[a]	21.5±2.4	13.7
	100	38.5±6.7	31.4±6.6	22.3
Alkannin	50	64.9±7.3	60.9±7.6	7.9
	100	70.6±6.3	57.2±3.6	21.3
Phenylbutazone	50	36.0±3.8	31.2±3.6	11.1

[a]Mean±S.E. (n=6).

ages (50 and 100 μg) of topical application. The reason for the lack of dose-response relationship could be that a 50-μg dose produces a maximal pharmacological response.

The results of the present experiments showed that there is no significant difference in the anti-inflammatory activity between shikonin and alkannin. It is concluded that the configuration of the hydroxy group on the side chain has little influence on the anti-inflammatory activity as well as on the antibacterial activity (5).

TABLE 2. Effects of Shikonin, Shikalkin, and Alkannin on Edema Formation after Thermal Injury (70°, 30 sec) in Rats

Test compound	Topical dosage (μg)	Intensity of edema (%)		Anti-edema activity (%)
		Control	Treatment	
Shikonin	50	83.4±4.6[a]	65.1±4.6*[b]	22.0
	100	105.2±6.7	83.5±5.8*	20.6
Shikalkin	50	80.2±4.3	59.3±4.7**	26.7
	100	80.8±10.1	64.2±8.5	23.2
Alkannin	50	103.0±9.5	81.0±7.1	21.3
	100	88.3±4.3	69.2±5.3*	21.7
Phenylbutazone	50	78.3±9.1	47.8±6.6*	38.9

[a]Mean±S.E. (n=10 or 12).

[b]The asterisks, * and **, indicate statistically significant differences at 5% and 1% level, respectively.

EXPERIMENTAL

PREPARATION OF ALKANNIN, SHIKALKIN, AND SHIKONIN.—Dried roots of *A. tinctoria* were extracted with $CHCl_3$. The $CHCl_3$ layer was treated with 5% NaOH to hydrolyze alkannin derivatives. The aqueous phase containing alkannin was adjusted to pH 3 with 1N HCl and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$, and evaporated in vacuo. The residue was subjected to preparative tlc (silica gel, $CHCl_3$) to isolate the red pigment, which was recrystallized from C_6H_6 and identified as alkannin by tlc, ir, and cd spectral comparisons with an authentic sample.

Shikalkin was isolated from dried roots of *Arnebia euchroma* (Royle) Johnst. (Boraginaceae), imported from China, by a method similar to that used for the isolation of alkannin.

Shikonin was prepared by hydrolyzing acetylshikonin which was isolated from callus tissues of *Echium lycopsis* L. (Boraginaceae).

The herb specimen of *E. lycopsis* and the root samples (crude drugs) of *A. tinctoria* and *A. euchroma* are preserved in the Herbarium of the Medicinal Plant Garden of Kyoto University.

Recently, we have found that the pigment components isolated from the roots or callus cultures of *L. erythrorhizon* as well as *E. lycopsis* consisted of both shikonin and alkannin in various ratios depending upon the esterified derivatives (10, 11). As for the purity of the samples used in the present experiments, the pigments isolated from the callus cultures of *E. lycopsis* and the roots of *A. tinctoria* were estimated to be a mixture of shikonin and alkannin in the ratios of 99:1 and 2:98, respectively, by cd measurements (10).

PREPARATION OF OINTMENT FOR BIOASSAY.—The ointment was prepared from sesame oil (918 g), beeswax (147 g), and white Vaseline (478 g) according to the method of Hayashi (6). The red pigment (shikonin, shikalkin, or alkannin) was homogeneously mixed with the ointment at a concentration of 0.1 and 0.2% (w/w).

BIOASSAY OF CAPILLARY PERMEABILITY.—Animals used were male albino rats of the Wistar strain, 140-180 g in body weight. Each dose group consisted of six rats. After careful shaving of the abdomen, each animal received a total of four applications of ointment (50 mg each) at two symmetrical sites on each side of the mid-line. Thirty minutes after the application of the ointment with or without the pigment, 4% pontamine sky blue 6BX solution (100 mg dye/kg body weight) was injected intravenously into the rats, followed by an intradermal injection of 0.05 ml of 0.331% histamine dihydrochloride solution. The animals were sacrificed 50 min after injection, and the skin at the shaved area was excised. According to the method of Judah and Willoughby (12), the skin tissues at the histamine-injected sites were cut out with a steel punch of 1.6 cm in diameter, cut into small pieces with scissors, then frozen with liquid nitrogen. The frozen tissue was placed in a small cup and crushed with a mallet; the pulverized tissue was transferred to a centrifuge tube and treated with 5 ml of 5% trichloroacetic acid for 15 h. The suspension was centrifuged at $500 \times g$, and the lipids in the pellet were removed with 5 ml of 50% EtOH at room temperature and further with 5 ml of absolute EtOH at 70° for 5 min, three times. The precipitated material was extracted with 3 ml of 25% aqueous pyridine at 80° for 40 min. After extraction with 2 ml of $CHCl_3$, the pyridine layer containing pontamine sky blue 6BX was measured colorimetrically at 600 nm. The anti-inflammatory activity was expressed as the percentage of inhibition of dye leakage.

BIOASSAY OF THERMAL EDEMA.—The procedure employed was a slight modification of the methods of Sevitt (13) and of Unger *et al.* (14). The animals used were female albino rats of the Wistar strain weighing 115 to 125 g. Two-and-a-half hours before thermal injury, the back of the rat was carefully shaved with electric clippers and completely depilated with softening cream (EBACREAM, Tanabe Pharmaceutical Co., Ltd.). Each animal received a total of six thermal injuries at three sites on both sides of the mid-line of the depilated skin. Thermal injury was produced by bringing the flat tip of a hot steel rod (1.0 cm in diameter) heated to 70° into firm contact with the depilated skin for 30 sec. Each ointment (50 mg) with or without the pigment was applied to each marked site 30 min before thermal injury. The same numbers of treatment and control sites were allocated at random to the opposite sites of the mid-line. Two hours after injury, the depilated area was excised and pinned to a board. The injured sites were cut out with a steel punch (1.6 cm in diameter) and weighed immediately.

A minimum of ten test and ten control sites were measured for each experiment, and the results were expressed as a mean of the intensity of edema, $I=(W_e-W_c)100/W_c$, where W_e is the weight of the edematous site and W_c is the weight of the corresponding control site. The anti-edema activity was expressed in terms of the percentage of inhibition of edema, $(I_c-I_t)100/I_c$, where I_c is the intensity of edema in the control animals and I_t is that in the animals treated with the pigment.

STATISTICAL EVALUATION.—Differences between controls and treatments in pharmacological experiments were analyzed by Student's *t* test.

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