Comparative Study on the Accelerative Effect of "Koushikon" and "Nanshikon" and Their Constituents on Proliferation of Granuloma Tissue in Rats

Yukihiro OZAKI,^{*,a} Akiko OHNO,^b Ken-ichi ABE,^b Yuji SAITO^b and Motoyoshi SATAKE^a

National Institute of Hygienic Sciences,^a 18-1, Kamiyoga 1-chome, Setagaya-ku, Tokyo 158, Japan and Chinese Traditional Medicine Research Laboratory, Kanebo Ltd.,^b 5-90, Tomobuchi-cho 1-chome, Miyakojima-ku, Osaka 534, Japan.

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This study was carried out to compare the accelerative effect in ether extracts of "Koushikon" and "Nanshikon" on proliferation of granuloma tissue in rats, and to elucidate this effect on optical isomer of naphthoquinone derivatives in those extracts. The content of total naphthoquinone derivatives in the ether extracts of Koushikon and Nanshikon were found to be 56.1% and 25.4%. Among naphthoquinone derivatives, Koushikon contained mostly acetyl derivative and Nanshikon mostly teracryl derivative. The percentage of *R*-type (shikonin-type) in total naphthoquinone derivatives of the extracts was 85.5% and 3.8%. Each ether extract showed a dose-dependent acceleration on the cotton pellet-induced granuloma formation. Comparison with corresponding doses containing the same quantity of naphthoquinone derivatives showed the accelerative potency of ether extracts of Koushikon and Nanshikon to be about the same. The result suggests that the accelerative effect on proliferation of granuloma tissue depends primarily on the total content of naphthoquinone derivatives, and not on the ratio of the optically active isomers.

Keywords *Lithospermum erythrorhizon*; *Macrotomia euchroma*; granuloma tissue; naphthoquinone derivative; optical isomer

In Japan "Shikon" has been used as a material to prepare an ointment called "Shiunko," which is frequently used for the treatment of wounds and burns.¹⁻³⁾

Two kinds of Shikon are known and available in Chinese markets. One type is called "Koushikon," which is the root of *Lithospermum erythrorhizon* SIEB. et ZUCC. and the other is called "Nanshikon," which is the root of *Macrotomia euchroma* PAULS. Only Koushikon has been used in Japan and it is recognized in the official Japanese Pharmacopoeia (Twelfth Edition).

Several pharmacological activities of Koushikon, Nanshikon and their constituents, shikonin, alkannin and acetylshikonin have been reported: bacteriocidal action, antitumor activity and promotion of the growth of granuloma tissue.³⁻¹⁰⁾ The constituents of Koushikon and Nanshikon were reported to be derivatives of shikonin but no detailed investigation of their absolute configurations was made. It was reported that Koushikon seemed to contain shikonin derivatives (*R*-configuration) and Nanshikon to contain alkannin derivatives (*S*-configuration).¹¹⁾

The present study was carried out to compare the accelerative effect of Koushikon and Nanshikon on proliferation of granuloma tissue in rats and to elucidate this effect on their own optically active constituents in both crude drugs.

Experimental

Ether Extracts of Koushikon and Nanshikon Dried roots of Koushikon and Nanshikon, (collected in the Chinese market in 1989, Mikuni Co., Ltd.), and Koushikon (bought in the Japanese market, Kinokuniya-kanyakyoku) ("Shikon") were refluxed with ether three times for 1 h each time.

The solution was filtered through filter paper and evaporated to give the ether extract under vacuum.

Quantity of Total Naphthoquinone Derivatives in the Ether Extracts The ether extracts were dissolved in ethanol, then 2.5% potassium hydroxide solution was added and shaken vigorously. The absorbance of the ethanol solutions was measured at 620 nm (Hitachi, model 200-10) and the total contents of naphthoquinone derivatives were calculated as shikonin.

Identification and Quantity of Naphthoquinone Derivatives in the Ether

Extracts The ether extracts were dissolved in methanol and naphthoquinone derivatives in them were determined by HPLC as described below. An octadecyl silica column (Inertsil ODS-2, 4.6 mm × 250 mm, GL Science Co., Ltd.) and mobile phase of phosphate buffer (0.2 M, pH 2.1): methanol (24:76) were used. The flow rate was 0.8 ml/min at a column temperature of 40°C and the column eluate was monitored at 520 nm. Identified peaks and their retention times were shikonin (or alkannin) (13.4 min), β -hydroxyisovaleryl derivative (19.2 min), acetyl derivative (21.0 min), teracryl derivative (33.2 min), a mixture of deoxy and isobutyl derivative (42.8 min), β , β -dimethylacryl derivative (57.3 min), α -methyl-*n*-butyl derivative (59.5 min) and isovaleryl derivative (61.4 min).

Each naphthoquinone derivative in the ether extracts was obtained by separation of the corresponding peak with HPLC under the conditions described above, and was confirmed by secondary ion mass spectrometry (SIMS) and ¹H-NMR spectral data. The composition of naphthoquinone derivatives in the extracts was estimated by the ratio of their peak area.

Identification of Absolute Configurations of Shikonin, Alkannin and Their Derivatives in the Ether Extracts The ether extracts were dissolved in ether and shikonin and alkannin, its enantiomer, in them were determined by HPLC as described below. An optically active column (Chiralcel OJ, 4.6 mm × 250 mm, Daisel Kagaku Kogyo Co., Ltd.) and a mobile phase of *n*-hexane:2-propanol:acetic acid = 95:5:0.3 were used. The flow rate was 1.0 ml/min at a column temperature of 40°C and the column eluate was monitored at 273 nm. The retention times of shikonin and alkannin were 13.5 and 15.8 min, respectively. Both constituents were prepared by separation with HPLC under the conditions described above, and were confirmed by circular dichroism (CD), SIMS and ¹H-NMR spectral data. The ratio of shikonin and alkannin was estimated by the ratio of their peak area.

The ratio of *R*-type and *S*-type in total naphthoquinone derivatives was determined as their hydrolyzate, shikonin or alkannin by HPLC. That is, 1 N potassium hydroxide solution was added to the ether extracts which were then shaken vigorously. Then 1 N hydrochloric acid was added to each solution, and the solutions were extracted with ether. The products, shikonin (total of *R*-type) and alkannin (total of *S*-type) in the ether extracts were determined by HPLC under the conditions described above.

Cotton Pellet-Induced Granuloma Formation Test The ether extracts were dissolved in 1% Tween 80 solution, and each solution was topically applied to a cotton pellet.¹⁰⁾

Male Wistar rats weighing about 250 g were anesthetized with ether. After sterilizing with 2% acrinol, the back skin was cut and a sterile cotton pellet about 20 ± 1.0 mg, to which 0.1 ml ether extract solution had been topically applied, was implanted subcutaneously into the shoulder. Control rats were treated similarly, except that they were implanted with a cotton pellet to which 0.1 ml 1% Tween 80 solution had been applied.

The cotton pellets together with the granuloma were removed from the

rats after 10 d.^{4,10)} The increment in dry weight was obtained as a measure of granuloma formation.

Statistical Analysis Data were expressed as the mean value $\pm$ standard error (%). All results were analyzed for variance by Bartlett's method. Significant differences were subsequently examined by Duncan's method.

Results

The Yield and Total Content of Naphthoquinone Derivatives in the Ether Extracts The yield and total content of naphthoquinone derivatives in the ether extracts of three kinds of Shikon are shown in Table I. The yield of the ether extract of Koushikon was larger than those of Shikon and Nanshikon. The total content of naphthoquinone derivatives in the ether extract of Koushikon was also larger: about twice that of Nanshikon and about one and a half times that of Shikon. The total content of naphthoquinone derivatives in the crude drugs of Koushikon was about three times that of Nanshikon and about twice that of Shikon.

Composition of Naphthoquinone Derivatives in the Ether Extracts The composition of naphthoquinone derivative in the ether extracts is shown in Table II. Shikonin content of Koushikon, Nanshikon and Shikon was 4.2%, 1.8% and 1.1%, respectively, while alkannin content was 0.6%, 0.4% and 2.2%.

Koushikon contained mostly acetyl derivative, Nanshikon contained teracryl derivative, and Shikon contained isovaleryl and α -methyl-*n*-butyl derivative. Tetracryl derivative was only found in Nanshikon.

Composition of Optically Active Isomer of Naphthoquinone Derivatives in the Ether Extracts Composition of shikonin (*R*-configuration, *R*-type) and alkannin (*S*-configuration, *S*-type) in the ether extracts of Koushikon, Nanshikon and Shikon is shown in Table III.

TABLE I. Yield (%) of Ether Extracts, Total Content of Naphthoquinone Derivatives in Ether Extracts and in Crude Drugs

Content (%)	Koushikon	Nanshikon	Shikon
Ether soluble extract ^{a)} in crude drugs	4.0	2.9	3.4
Total content of naphthoquinone ^{b)} derivatives in ether extract	56.1	25.4	38.1
Total content of naphthoquinone ^{c)} derivatives in crude drugs	2.2	0.7	1.3

c) Calculated from a) and b).

TABLE II. Composition of Naphthoquinone Derivatives in Ether Extracts

Composition of naphthoquinone derivatives in ether extract (%)	Koushikon	Nanshikon	Shikon
Shikonin	4.2	1.8	1.1
Alkannin	0.6	0.4	2.2
β -Hydroxyisovaleryl derivative	17.5	6.0	1.3
Acetyl derivative	38.7	5.1	17.2
Teracryl derivative	N.D.	37.2	N.D.
Deoxy + isobutyl derivative	18.1	19.4	5.1
β , β -Dimethylacryl derivative	3.2	9.4	3.4
α -Methyl- <i>n</i> -butyl derivative	17.7	16.2	30.8
Isovaleryl derivative		4.6	38.8

N.D.: not detected.

The percentage of *R*-type in total naphthoquinone derivatives of these extracts was 85.5%, 3.8% and 2.8%, respectively, while the percentage of *S*-type in total naphthoquinone derivatives was 14.5%, 96.2% and 97.2%.

The ratio of *R*-type and *S*-type content in total naphthoquinone derivatives of Nanshikon was about the same as that of Shikon.

Effects of the Ether Extracts on Cotton Pellet-Induced Granuloma Formation The ether extracts showed a dose-dependent acceleration on cotton pellet-induced granuloma formation. The extracts of Koushikon (at 0.26% and 0.87%), Nanshikon (at 0.58% and 1.93%) and Shikon (at 0.13%, 0.39% and 1.29%) significantly accelerated this formation, but those of Koushikon (at 0.09%) and Nanshikon (at 0.19%) did not show the acceleration.

Comparison with the corresponding doses containing the same quantity of naphthoquinone derivatives showed the accelerative potency of ether extracts of Koushikon and Nanshikon to be about the same. The extract of Shikon was somewhat stronger than either of the others. These results are shown in Table IV.

Discussion

In the ether extracts of the three materials, Koushikon contained mainly acetyl derivative, Nanshikon contained teracryl derivative, and Shikon contained isovaleryl and α -methyl-*n*-butyl derivative. Teracryl derivative was only found in Nanshikon. The percentage of *R*-type (shikonin-type) in those extracts was 85.5%, 3.8% and 2.8%, respectively, while the percentage of *S*-type (alkannin-type) was 14.5%, 96.2% and 97.2%.

Tsukada *et al.* reported that many samples of Lithospermi Radix (Shikon, collected in Japan, China, Hong Kong and Korea markets) were assayed for their constituents of

TABLE III. Composition of Optically Active Isomer of Naphthoquinone Derivatives in Ether Extracts

Composition of optically active isomer in naphthoquinone derivatives (%)	Koushikon	Nanshikon	Shikon
<i>R</i> -Type	85.5	3.8	2.8
<i>S</i> -Type	14.5	96.2	97.2

TABLE IV. Effects of Ether Extracts on Proliferation of Granulation Tissue in Rats

Compound	Dose (%)	No. of animals	Proliferation of granuloma tissue (%)
Control		10	116.9 $\pm$ 3.4
Koushikon	0.09	6	115.2 $\pm$ 2.0
	0.26	6	127.0 $\pm$ 2.3 ^{a)}
	0.87	6	141.8 $\pm$ 4.5 ^{b)}
Nanshikon	0.19	6	121.7 $\pm$ 2.5
	0.58	6	129.4 $\pm$ 3.0 ^{a)}
	1.93	6	144.5 $\pm$ 3.0 ^{b)}
Shikon	0.13	6	129.5 $\pm$ 4.2 ^{a,c)}
	0.39	6	145.7 $\pm$ 2.4 ^{b,d)}
	1.29	6	162.6 $\pm$ 4.9 ^{b,d)}

a, b) Significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively. c) Significantly different from a low dose of Koushikon at $p < 0.05$. d) Significantly different from a medium dose and a high dose of Koushikon and Nanshikon at $p < 0.01$, respectively.

naphthoquinone derivatives; all the samples of Nanshikon extracts tested contained the teracryl derivative but none of Koushikon contained it.¹¹⁾ They also showed that Koushikon contained a major quantity of *R*-type with a smaller amount of *S*-type, while Nanshikon contained a major amount of *S*-type with a minor quantity of *R*-type. They suggested that some Koushikon might be the root of some other boraginaceous species.

The plants which are the morphological origins of those used in this experiment are: Koushikon is the root of *Lithospermum erythrorhizon* SIEB. et ZUCC., Nanshikon is the root of *Macrotomia euchroma* PAULS., and Shikon is probably the root of *Lithospermum erythrorhizon* SIEB. et ZUCC. But, judging from its constituents and the biological activity mentioned below, the latter might be the root of another boraginaceous species as shown Tsukada *et al.*

It was found in this study that the ether extracts of Koushikon, Nanshikon and Shikon showed a dose-dependent acceleration on the cotton pellet-induced granuloma formation. Comparison with the corresponding doses containing the same quantity of naphthoquinone derivatives showed the accelerative potency of the extracts of Koushikon and Nanshikon to be about the same, and that of Shikon to be somewhat stronger. It has been reported that shikonin and alkannin showed bacteriocidal action, antitumor activity and an accelerative effect on proliferation of granuloma tissue, and that the potency of these effects induced by the two compounds was about the same.³⁻⁹⁾ It has also been reported that shikonin and acetylshikonin showed a dose-dependent acceleration on cotton pellet-induced granuloma formation of about the same potency.⁴⁾

From these results, it is suggested that the acceleration of proliferation of granuloma tissue induced by the ether extracts of Koushikon, Nanshikon and Shikon is due to the total shikonin derivatives contained in the extracts, and that the difference of constituents and their absolute configurations (*R*-type or *S*-type, *i.e.*, the configuration of the hydroxy group on side chain) may not be important in producing the acceleration of proliferation of granuloma tissue.

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