Accelerative Effect of “Nanshikon” and Its Constituents on the Proliferation of Granulation Tissue in Rats

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The present study was carried out to examine the accelerative effect of the ether extract obtained from “Nanshikon” on the proliferation of granulation tissue induced by cotton pellet in rats and to elucidate its active principles. Among naphthoquinone derivatives, the ether extract contained mostly teracrylshikonin. At corresponding doses based on the contents of the naphthoquinone derivatives in the ether extract, the accelerative potencies of teracrylshikonin, β,β -dimethylacrylshikonin, and a mixture of α -methyl-*n*-butylshikonin and isovalerylshikonin were about the same. Also, the accelerative potencies of these compounds were somewhat weaker than that of the ether extract. From these results, it is suggested that the accelerative effect of the ether extract on the proliferation of granulation tissue is mainly due to teracrylshikonin, β,β -dimethylacrylshikonin and a mixture of α -methyl-*n*-butylshikonin and isovalerylshikonin contained in the ether extract, and the accelerative effect induced by ether extract might be an additive effect of these naphthoquinone derivatives.

Key words *Macrotomia euchroma*; teracrylshikonin; β,β -dimethylacrylshikonin; α -methyl-*n*-butylshikonin; isovalerylshikonin; granulation tissue proliferation

We previously reported that the ether soluble extracts of “Koushikon”, which is the root of *Lithospermum erythrorhizon* SIEB. et ZUCC., and “Nanshikon”, which is the root of *Macrotomia euchroma* PAULS., produced an accelerative effect on the proliferation of granulation tissue in rats, and that the accelerative potency induced by both extracts was about the same, although the kind of constituents and the ratio of optical isomer active constituents in the ether extracts were different.^{1,2)}

Next, we showed that shikonin, alkannin and acetylshikonin, isolated from both ether extracts, produced an accelerative effect on the proliferation of granulation tissue and the accelerative potency induced by these compounds was about the same.

Further, it was suggested that the absolute configuration of the hydroxy group in the sidechain and the presence of an acetyl group on the hydroxy group of the sidechain of shikonin or alkannin may not be important in producing the accelerative effect.³⁾

Although many pharmacological studies about Koushikon and its constituents have been reported,^{4–12)} there have been very few studies about the effect of Nanshikon and its constituents on granuloma formation.

The present study was carried out to study the accelerative effect of the ether extract of Nanshikon on the proliferation of granulation tissue in rats and to elucidate the active constituents contained in this extract.

MATERIALS AND METHODS

Ether Extract of “Nanshikon” Dried root of “Nanshikon” (collected in a Chinese market in 1989, Mikuni Co., Ltd.) was extracted with ether three times under reflux for 1 h.

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

Quantitative Analysis of Total Naphthoquinone Deriva-

tives in the Ether Extract The ether extract was dissolved in ethanol, then 2.5% potassium hydroxide solution was added and shaken vigorously. The absorbance of the ethanol solution was measured at 620 nm (Hitachi, model 200-10) and the total content of naphthoquinone derivatives was calculated as shikonin.

Identification and Quantification of Naphthoquinone Derivatives in the Ether Extract The composition of naphthoquinone derivatives in the ether extract was determined by HPLC as described below. An octadecyl silica column (Inertsil ODS-2, 4.6 mm $\times$ 250 mm, GL Science Co., Ltd.) and a mobile phase 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 were shikonin or alkannin, β -hydroxyisovalerylshikonin, acetylshikonin, teracrylshikonin, a mixture of deoxyshikonin and isobutylshikonin, β,β -dimethylacrylshikonin, and a mixture of α -methyl-*n*-butylshikonin and isovalerylshikonin.

The quantity of naphthoquinone derivatives in the extract was estimated by the ratio of their peak area.

Isolation of Shikonin and Alkannin Optically pure shikonin ((*R*)-configuration, (*R*)-type) and alkannin ((*S*)-configuration, (*S*)-type) were separated from commercial shikonin (Funakoshi Co., Ltd.) by HPLC under the conditions described below.

An optically active column (Chiralcel OJ, 4.6 mm $\times$ 250 mm, Daicel Chemical Industries, 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. The compounds were recrystallized from ether, and the structures were confirmed by circular dichroism (CD), secondary ion mass spectrometry (SIMS) and ¹H-NMR spectral data.

The ratio of (*R*)-type and (*S*)-type isomers in total

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

Isolation of Naphthoquinone Derivatives from the Ether Extract From the ether extract of Nanshikon, naphthoquinone derivatives were isolated by collecting the corresponding peaks on HPLC under the conditions described below. An octadecyl silica column (YMC-Pack ODS-5-D, 20 mm × 250 mm, YMC Co., Ltd.) and a mobile phase of 75% CH₃CN were used. The flow rate was 9.0 ml/min at room temperature, and the column eluate was monitored at 520 nm. The compounds were recrystallized from ether and the structures were confirmed by SIMS and ¹H-NMR spectral data.

Cotton Pellet-Induced Granuloma Formation Test The ether extract and naphthoquinone derivatives were dissolved in 1% Tween 80 solution, and each solution was topically applied to a cotton pellet.¹⁻³⁾

Male Wistar rats weighing about 250 g were used. The rats were anesthetized with ether. After being sterilized in 2% acrinol, the back skin was cut and a sterile cotton pellet about 20 ± 1.0 mg, to which 0.1 ml test 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 granuloma, were removed from the rats after 10 d. The pellets were dried in an incubator at 60 °C for about 24 h until a constant weight was obtained. The incremental change in dry weight was obtained as a measure of granuloma formation.

The results are expressed as the percent increase in dry weight due to granuloma formation (%), as compared with the initial cotton pellet weight.

Statistical Analysis Data are expressed as the mean value ± standard error (%). The statistical significance of difference between the groups was determined by Duncan's multiple range test.

RESULTS

Yield of the Ether Extract, Total Content of Naphthoquinone Derivatives and Composition of Optically Active Isomers in the Ether Extract The yield of the ether extract, the total content and the composition of the optically active isomer of naphthoquinone derivatives in the ether extract obtained from Nanshikon are shown in Table 1.

The yield of the ether extract from Nanshikon and the total content of naphthoquinone derivatives in the ether extract were 2.9% and 25.4%, respectively. The percentages of (*R*)-type and (*S*)-type isomers in the total naphthoquinone derivatives were 3.8% and 6.2%, respectively.

Composition of Naphthoquinone Derivatives in the Ether

Table 1. Yield (%) of Ether Extract, Total Content (%) of Naphthoquinone Derivatives and Composition of Optically Active Isomers in Ether Extract Obtained from Nanshikon

Composition	Content (%)
Yield % of ether soluble extract in Nanshikon	2.9
Yield % of total content of naphthoquinone derivatives in ether extract	25.4
Composition % of optically active isomer on naphthoquinone derivatives in ether extract	
(<i>R</i>)-type	3.8
(<i>S</i>)-type	97.2

Extract The ether extract obtained from Nanshikon contained many kinds of naphthoquinone derivatives, and the chemical structures of naphthoquinone derivatives obtained from the ether extract of Nanshikon are shown in Fig. 1.

Among these naphthoquinone derivatives, most were teracryl derivatives and only a few were alkannin.

Effect of the Ether Extract and the Naphthoquinone Derivatives on Cotton Pellet-Induced Granuloma Formation The ether extract obtained from Nanshikon showed a dose-dependent acceleration effect on cotton pellet-induced granuloma formation (at concentration of 0.58 and 1.93%).

The effect of the naphthoquinone derivatives was tested at concentrations corresponding to the amounts in the ether extract, at 1.93%. Teracrylshikonin, at 0.18%, β,β-dimethylacrylshikonin, at 0.05%, and a mixture of α-methyl-*n*-butylshikonin and isovalerylshikonin, at 0.1%, showed an accelerative effect on cotton pellet-induced granuloma formation, but the other naphthoquinone derivatives did not show any significant accelerative effect. The potency of accelerative effects induced by these compounds was somewhat weaker than that of the ether extract.

These results are shown in Table 3.

DISCUSSION

Concerning the naphthoquinone derivatives and their optically active isomers in the ether extract obtained from Nanshikon, they contained mainly teracrylshikonin and small amounts of shikonin and alkannin. The percentages of (*R*)-type (shikonin-type) and (*S*)-type (alkannin-type) isomers in the extract were 3.8% and 96.2%, respectively.

Tsukada *et al.* reported that many samples of *Lithospermi Radix*, collected in Japan, China, Hong Kong and Korea markets, were assayed for their naphthoquinone derivatives; all the samples of Nanshikon extracts tested contained the teracryl derivative but none of Koushikon contained it. And also, they showed that Nanshikon contained a major amount of (*S*)-type with a minor quantity of (*R*)-type, whereas Koushikon contained a major quantity of (*R*)-type with a smaller amount of (*S*)-type.¹²⁾

From these results, concerning the morphologically original plant of Nanshikon used in this experiment, the medicinal plant is the root of *Macrotomia euchroma* PAULS ("Nanshikon").

Fig. 1. Chemical Structures of Naphthoquinone Derivatives Obtained from the Ether Extract of Nanshikon

Table 2. Composition of Naphthoquinone Derivatives in Ether Extract Obtained from Nanshikon

Composition of naphthoquinone derivatives in ether extract	Content (%)
Shikonin	1.8
Alkannin	0.4
β -Hydroxyisovalerylshikonin	6.0
Acetylshikonin	5.1
Teracrylshikonin	37.2
Deoxyshikonin + isobutylshikonin	19.4
β, β -Dimethylacrylshikonin	9.4
α -Methyl- <i>n</i> -butylshikonin + isovalerylshikonin	20.8

In the present study, it was found that the ether extract obtained from Nanshikon showed a dose-dependent acceleration effect on cotton pellet-induced granuloma formation. At doses corresponding to the yields of contained naphthoquinone derivatives, the accelerative potencies of teracrylshikonin, β, β -dimethylacrylshikonin, and a mixture of α -methyl-*n*-butylshikonin and isovalerylshikonin were about the same, but their potencies were somewhat weaker than that of the ether extract, and other naphthoquinone derivatives did not show any significant acceleration.

It has been reported that shikonin, alkannin and acetylshikonin showed dose-dependent acceleration on

Table 3. Effects of Ether Extract and Naphthoquinone Derivatives Obtained from Nanshikon on Proliferation of Granulation Tissue in Rats

Compounds	Dose (%)	Number of animals	Proliferation of granulation tissue (%)
Control		8	135.9 $\pm$ 5.8
Ether extract of Nanshikon	0.58	8	158.6 $\pm$ 9.5
Shikonin	1.93	8	189.0 $\pm$ 11.7 ^{a)}
Alkannin	0.002	8	167.6 $\pm$ 13.5 ^{b)}
Acetylshikonin	0.03	8	152.3 $\pm$ 15.6 ^{b)}
β -Hydroxyisovalerylshikonin	0.03	8	160.1 $\pm$ 6.9 ^{b)}
Teracrylshikonin	0.18	8	177.5 $\pm$ 13.2 ^{a,b)}
Deoxyshikonin + isobutylshikonin	0.1	8	167.7 $\pm$ 17.6 ^{b)}
β, β -Dimethylacrylshikonin	0.05	8	176.6 $\pm$ 12.8 ^{a,b)}
α -Methyl- <i>n</i> -butylshikonin + isovalerylshikonin	0.1	8	170.3 $\pm$ 10.4 ^{a,b)}

a) Significantly different from the control at $p < 0.05$. b) Not significantly different from the ether extract of Nanshikon.

cotton pellet-induced granuloma formation and their potencies are about the same. Also, the absolute configurations ((*R*)-type or (*S*)-type) of the hydroxy group in the sidechain of shikonin or alkannin and the presence of an acetyl group on the hydroxy group may not be important in producing the effect.³⁾

From these results, it is suggested that the acceleration

of the proliferation of granuloma tissue induced by the ether extract of Nanshikon is mainly due to teracrylshikonin, β,β -dimethylacrylshikonin, and a mixture of α -methyl-*n*-butylshikonin and isovalerylshikonin, while other naphthoquinone derivatives, especially shikonin, alkannin, acetylshikonin and other compounds might partly play a role in producing the accelerative effect, and the effect induced by these naphthoquinone derivatives might be an additive effect.

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