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Photochemical decomposition of alkannin/shikonin enantiomers

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Abstract

Alkannin/shikonin ((±)-5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone), isolated from *Macrotomia euchroma*, is sensitive to light exposure. A chloroform solution of alkannin/shikonin was exposed to sunlight for 1 month. The major photolytic product, a newly discovered naphthoquinone derivative, was isolated by adsorption chromatography. The chemical structure of the purified photolytic product was identified by NMR, MS and FTIR techniques to be (−)-5,8-dihydroxy-2-(1-hydroxy-3-oxo-4-methyl-4-pentenyl)-1,4-naphthoquinone, indicating that a photo-oxidation reaction had occurred.

Keywords: Alkannin/shikonin; Photochemical decomposition; *Macrotomia euchroma*; Photodegradation

1. Introduction

Alkannin and its enantiomer shikonin (Fig. 1), the main pharmacological components of Shikon, are present in three types of boraginaceous plants in different ratios (Ikeda et al., 1991). While *Alkanna tinctoria* and *Macrotomia euchroma* have greater content of alkannin, *Lithospermum erythrorhizon* has a higher content of shikonin. Two kinds of Shikon are known and available in the Orient: Ko-shikon is the root of *L. erythrorhizon*; the other type is Nan-shikon which is the root of *M. euchroma* (Sankawa et al., 1981). Most of the Shikon available on the market is the root of *M. euchroma* which is imported from China (Sankawa et al., 1977). As stated in the 'Greek Herbal of Dioscorides' in the 1st century, the root of *A. tinctoria* was used for wound healing (Papageorgiou, 1980). In Japan, Shikon has been used as a major ingredient to prepare Shiunko ointment, which is frequently used for the treatment of wounds, skin diseases, and burns (Hayashi, 1977a,b; Konoshima et al., 1989; Seto et al., 1992). Alkannin and shikonin show no significant difference in terms of therapeutical activities (Tanaka et al., 1986). The pharmacological activities of alkannin/shikonin include anti-inflammatory (Hayashi, 1977a; Tanaka et al., 1986), antibacterial (Tabata et al., 1975, 1982; Honda et al., 1988), wound healing (Hayashi, 1977a,b; Papageorgiou, 1978; Seto et al., 1992), and antitumor effects (Sankawa et al., 1977, 1981; Konoshima et al., 1989).

Although the pharmacological activities of

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alkannin/shikonin and their derivatives have been extensively studied, very little research has been performed on the chemistry of these compounds. Since most of the pharmaceutical preparations containing Shikon extract are for dermatological use, light exposure is an inevitable step during the treatment regimen. The objective of the present study, therefore, was to investigate the effect of light on the pharmacologically active components of *M. euchroma*, alkannin/shikonnin, and the identification of the major photolytic product.

2. Materials and methods

2.1. Materials

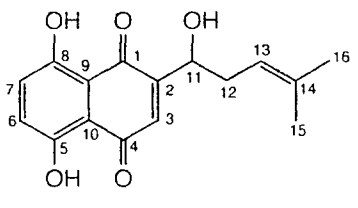
Acetonitrile and methanol (LC grade) were purchased from Lab-Scan (Dublin, Ireland). Dichloromethane (LC grade), silica gel 60, *n*-hexane and chloroform (extra pure grade) were purchased from E. Merck (Darmstadt, Germany). Cosmosil reversed-phase HPLC column was purchased from Nacalai Tesque (Japan). Dry roots of *M. euchroma* were purchased from a local herb store.

2.2. Preparation of alkannin / shikonin

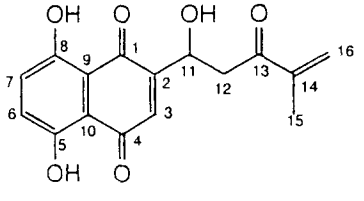
Alkannin/shikonin was prepared following the procedure of Tabata et al. (1982). Dried roots (3 kg) of *M. euchroma* were extracted with 25 l of *n*-hexane. The extraction process was repeated twice with 25 l of *n*-hexane. The combined *n*-hexane extract was evaporated using a Yamato vacuum rotary evaporator (Japan). The crude extract was dissolved in 1 N NaOH and filtered through a 90 mm Advantec filter paper (Toyo Roshi Kaisha, Japan). The solution was acidified with 0.5 N H_2SO_4 to pH 6 and re-extracted with *n*-hexane. The *n*-hexane layer was further concentrated using the vacuum rotary evaporator. The concentrate was chromatographed through an open column of silica gel 60 (15 × 15 cm). A gradient of *n*-hexane/chloroform of ratio from 80:20 to 50:50 was used as an eluent. The purified alkannin/shikonin (17 g) was recrystallized from dichloromethane and verified by its melting point. The specific rotation of the alkannin/shikonin was determined with a Jasco DIP-140 digital polarimeter. Spectrometric analysis of alkannin/shikonin was performed with Fourier transform infrared (FTIR) (KBr, Biorad Digilab FTS-40), MS (Finnigan TSQ 46C) and NMR ($CDCl_3$, Bruker AM-300 WB FT-NMR) techniques. Electron ionization (EI) mass spectra were recorded at 70 eV by direct probing into the ion source.

2.3. Analysis of photodegraded sample of alkannin / shikonin by HPLC

A 1 mg/ml chloroform solution of alkannin/shikonin was exposed to sunlight for 1 month. A Jasco 880-PU HPLC system equipped with a Linear 206 PHD photo-diode array detector was used to analyze this photodegraded sample on a Cosmosil $5C_{18}$-AR column (i.d. 4.6 mm × 25 cm) at 280 nm. A mixture of $CH_3CN/MeOH/H_2O$ (54.4:16.2:29.4) was used as the mobile phase at a flow rate of 0.5 ml/min.



Alkannin/shikonin



Major photolytic product (MPP)

Fig. 1. Chemical structures of alkannin/shikonin and MPP.

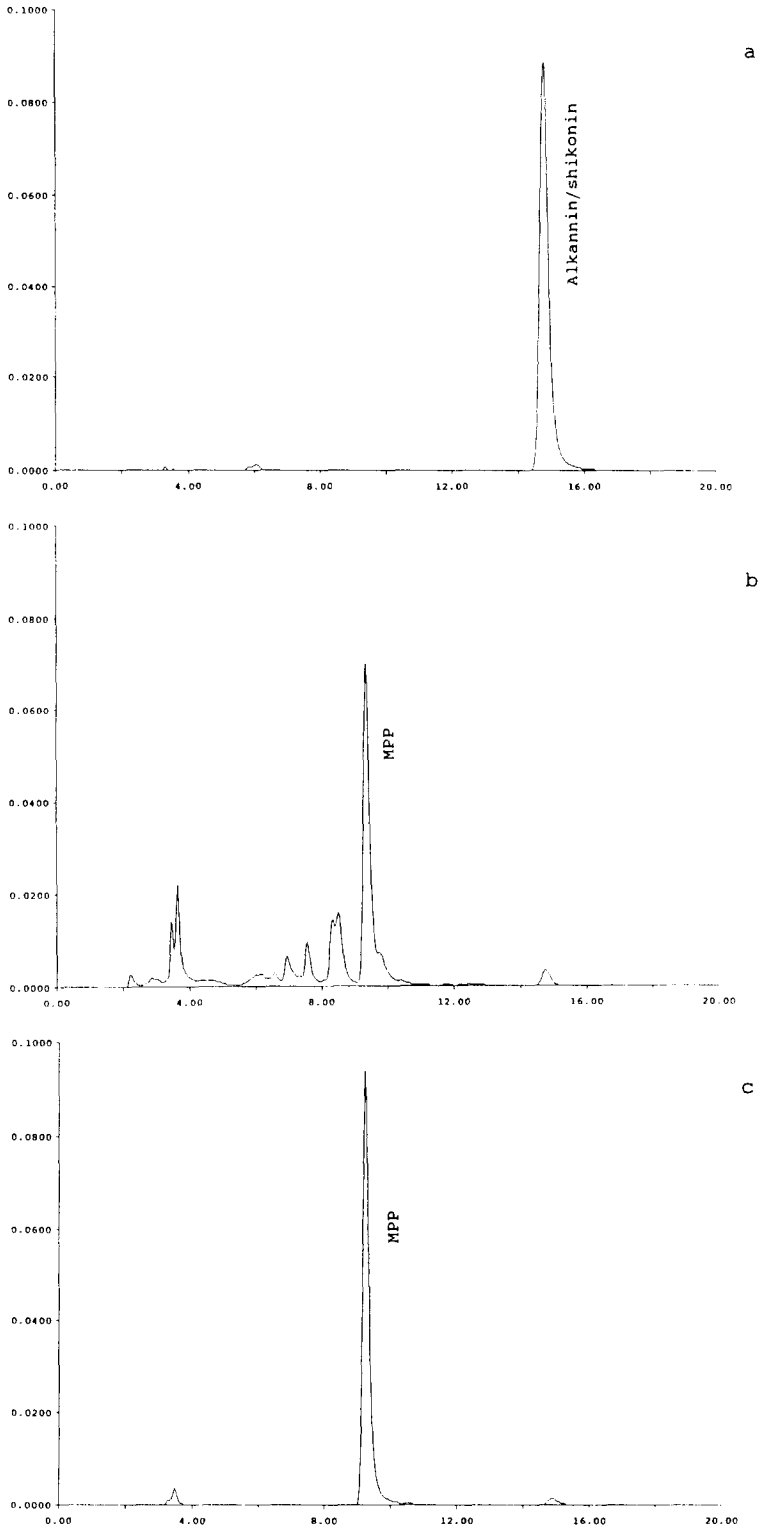


Fig. 2. HPLC chromatogram of alkannin/shikonin chloroform solution before exposure to sunlight (a), photodegraded chloroform solution of alkannin/shikonin (b), and purified MPP (c).

2.4. Isolation and identification of major photolytic product (MPP)

500 ml of the photodegraded alkannin/shikonin sample as described in the previous section was evaporated and chromatographed through an open column of silica gel 60 (2 × 15 cm). A gradient of *n*-hexane/chloroform of ratio from 80:20 to 50:50 was used to elute the MPP. HPLC analysis was used to monitor the progress of purification. The fractions that contained only the MPP were collected and were further concentrated with the vacuum rotary evaporator. About 18.5 mg of pure MPP was obtained after repetitive silica gel chromatography and recrystallization. Spectrometric analysis was performed on the purified degradant with NMR ($CDCl_3$), MS and FTIR (KBr) techniques to elucidate the chemical structure of the MPP.

3. Results and discussion

3.1. Purification of alkannin / shikonin

17 g of alkannin/shikonin were extracted from the dry roots of *M. euchroma* following the method of Tabata et al. (1982) with slight modification. The alkannin/shikonin had a melting point of 147–149°C. NMR, MS and IR analyses of the purified compound were performed. The spectra obtained corresponded with those in the literature (Cong, 1984; Inoue et al., 1985). The chemical structure of the purified compound was confirmed to be alkannin/shikonin.

The purified alkannin/shikonin had a $[\alpha]_D^{26}$ $-150°$ ($c = 1$, $CHCl_3$). The listed $[\alpha]_D^{20}$ of alkannin is $-226°$ in the Merck Index. Therefore, the red crystal we obtained is a mixture of alkannin/shikonin with an enantiomeric ratio of

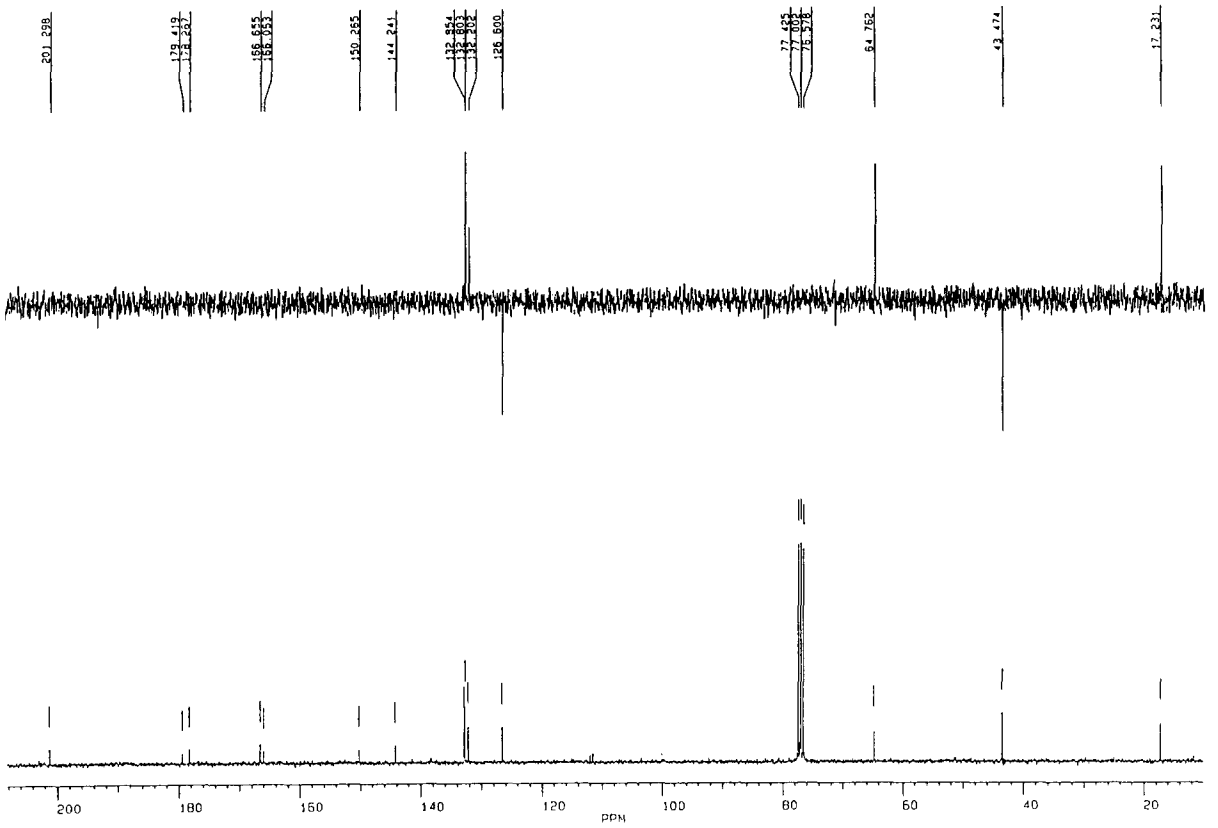


Fig. 3. DEPT (135°) spectrum of MPP. The top portion of the spectrum reveals five primary and tertiary carbons (up peaks at 132.85, 132.80, 132.20, 64.76 and 17.23 ppm) and two secondary carbons (down peaks at 126.60 and 43.47 ppm).

approx. 83:17. This material was used for all subsequent studies.

3.2. Photodegradation of alkannin / shikonin

The chloroform solution of alkannin/shikonin was exposed to sunlight for 1 month. This solution, before and after sunlight exposure, was subjected to HPLC analysis. Comparing these chromatograms, we noted that about 96% of the alkannin/shikonin had degraded under the experimental conditions (Fig. 2a,b). This result indicated that alkannin/shikonin was light labile. The main absorption peak in Fig. 2b constituted about 50% of the total area with a retention time of 9.5 min. We therefore designated this main absorption peak as the major photolytic product (MPP) and focused our attention on the identification of MPP.

3.3. Isolation and identification of MPP from the photodegraded alkannin / shikonin chloroform solution

Photodegraded alkannin/shikonin sample was subjected to silica gel chromatography to isolate the MPP. HPLC analysis of the purified MPP is shown in Fig. 2c. It was a red solid compound with a melting point of 165–169°C. It had a specific rotation of $[\alpha]_D^{26}$ −61° ($c = 1$, $CHCl_3$).

Spectrometric analyses were carried out on MPP to elucidate its chemical structure. The distortionless enhancement by polarization transfer (DEPT) (135°) and ^{1}H-NMR spectra are shown in Fig. 3 and 4. The data for alkannin/shikonin from Inoue et al. (1985) are summarized in Table 1 and were used as a reference to assign individual absorption peaks of MPP. Both alkannin/shikonin and MPP had 16 carbon absorption

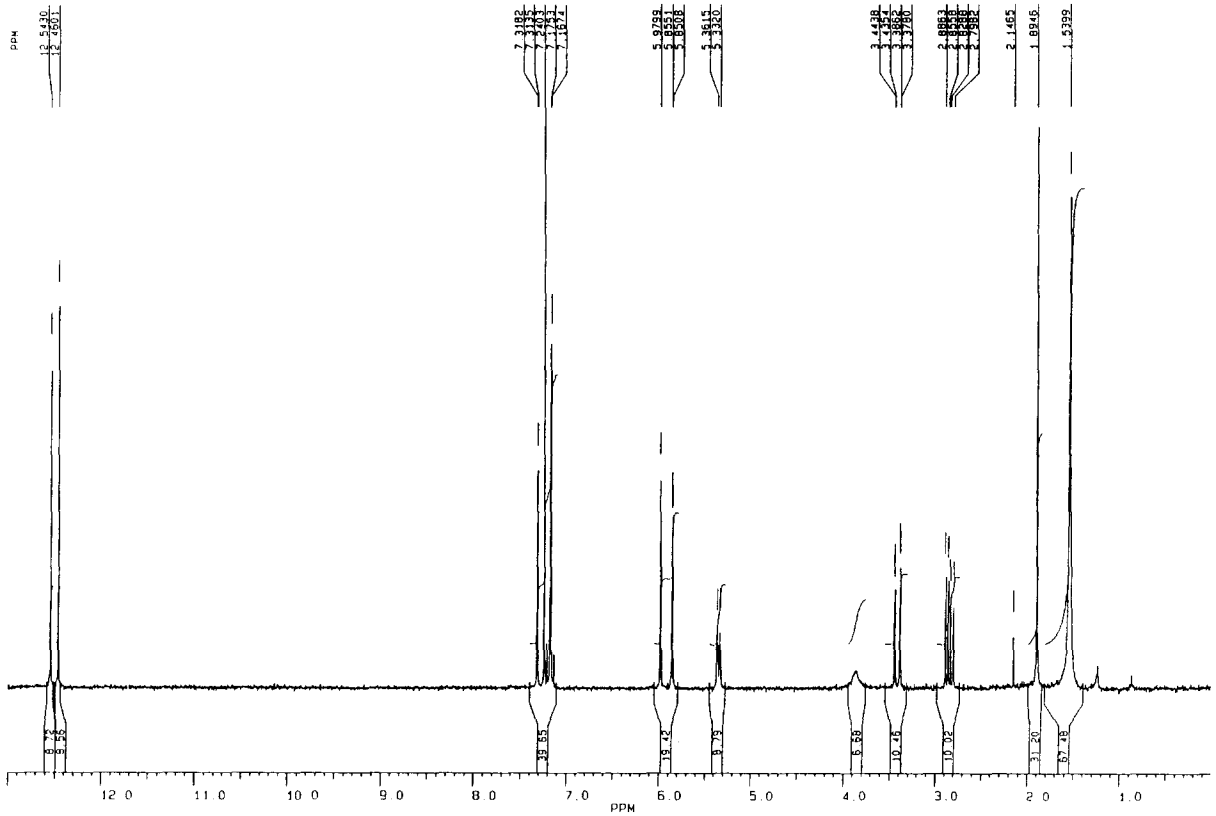


Fig. 4. ^{1}H-NMR spectrum of MPP.

peaks. There were no significant chemical shift differences between alkannin/shikonin and MPP from C1 to C10 on DEPT and ^{1}H-NMR. The results indicated that MPP had retained the base skeleton of 5,8-dihydroxy-1,4-naphthoquinone.

Whilst the 17.23 ppm peak in the DEPT spectrum indicates the retention of the methyl group at C-15 (Fig. 3), the absence of the 25.91 ppm chemical shift at C-16 of alkannin/shikonin (Table 1) suggests that the C-16 methyl group had changed. The characteristic alkene absorption peaks had shifted from 118.54 and 137.27 ppm to 126.60 and 144.24 ppm. Since the DEPT (135°) spectrum of MPP also revealed that the chemical shift at 126.60 ppm was a secondary carbon, the C=C double bond must have shifted from position C13–C14 of alkannin/shikonin to C14–C16 of MPP to have the secondary carbon absorption. The existence of the terminal -C=CH_2 between C14 and C16 could be further substantiated by the FTIR spectrum of MPP (Fig. 5). The strong absorption peak at 3077 cm^{-1} supports the existence of a terminal =CH_2 group at the side chain of MPP.

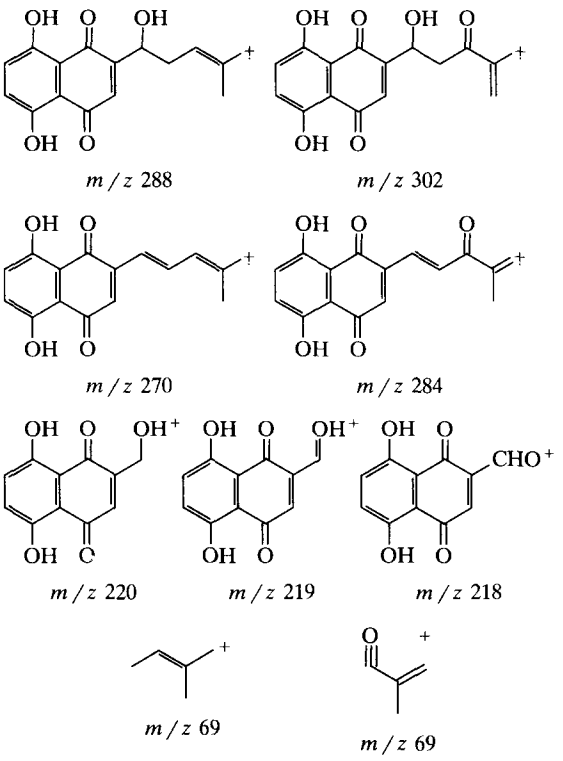
Table 1
^{13}C- and ^{1}H-NMR of alkannin/shikonin (Inoue et al., 1985)

Carbon no.	Alkannin/shikonin	
	^{1}H-NMR	^{13}C-NMR
1		179.66
2		151.47
3	H-3 7.16 d	131.91
4		180.44
5	OH-5 12.48 s	165.73
6	H-6 7.19 s	132.35
7	H-7 7.19 s	132.35
8	OH-8 12.58 s	165.09
9		112.09
10		111.60
11	H-11 4.91 ddd	68.43
12	H-12 2.35 br td 2.65 ddd	35.73
13	H-13 5.20 qut	118.54
14		137.27
15	H-15 1.65 br s	18.07
16	H-16 1.76 br s	25.91

br, broad; s, singlet; d, doublet; t, triplet; q, quartet; qut, quintet; m, multiplet.

Table 2
Comparison of EI mass spectral data of alkannin/shikonin (Cong, 1984) and MPP

Mass fragment	*m/z*	
	Alkannin/shikonin	MPP
M+	288	302
M−H_2O	270	284
Base peak	220	69
$C_{11}H_7O_5$	219	219
$C_{11}H_6O_5$	218	218



The comparison of the ion peaks of alkannin/shikonin (Cong, 1984) and MPP is summarized in Table 2. The molecular ion peak (*m/z* 302) is prominent. The molecular weight increment of 16 to the parent compound, alkannin/shikonin, suggests that an oxygen was incorporated into MPP. While the 64.76 ppm in the DEPT spectrum of MPP indicates the preservation of a hydroxyl group, the newly found chemical shift at 201.29 ppm, a distinct carbonyl absorption peak, thus confirms the introduction of an oxygen atom into alkannin/shikonin to

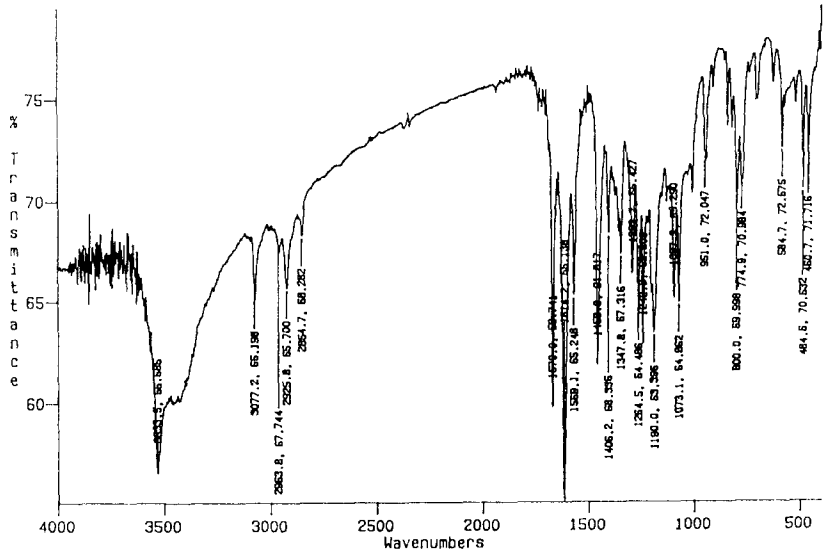


Fig. 5. FTIR spectrum of MPP. The newly formed 1670 and 3077 cm^{-1} peaks represent the formation of a carbonyl and a terminal $=CH_2$ group.

form MPP. The strong FTIR absorption peak at 1670 cm^{-1} also supports the formation of a carbonyl group. The strong base peak (m/z 69) intensity suggests the carbonyl group is located at the C-13 position of MPP, thus giving a C_4H_5O ion instead of C_5H_9 (a significant ion fragment from the parent compound). While the terminal $=CH_2$ group generally absorbs in the range of 110–115 ppm in ^{13}C-NMR, the electron-withdrawing effect of the carbonyl group at C-13 position had a strong downfield effect and shifted its absorption to 126.6 ppm.

The retaining of a hydroxyl group at the side chain of MPP was confirmed by the characteristic M – 18 ion peak (m/z 284) in the MS spectrum. The common significant ions of m/z 218, 219 and 220 of alkannin/shikonin (Cong, 1984) and MPP indicate that the hydroxyl group remains at the C-11 position. The chemical shift which changed from 68.43 to 64.76 ppm in the DEPT spectrum is due to the upfield effect of the carbonyl group at the C-13 position. Since there were two secondary carbons in the DEPT (135°) spectrum, that at 43.47 ppm should be located at the C-12 position. Therefore, The chemical structure of MPP was identified as (–)-5,8-dihydroxy-2-(1-hydroxy-3-oxo-4-methyl-4-pentenyl)-1,4-naphthoquinone (Fig. 1).

The photolytic reaction that leads to the formation of MPP is a photosensitized oxidation at

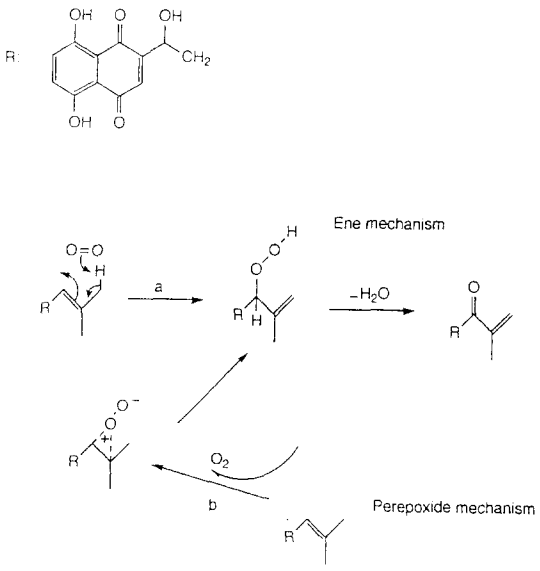


Fig. 6. Postulated photo-oxidation mechanism of alkannin/shikonin to MPP: ene mechanism (a); perepoxide mechamism (b).

the C-13 double bond. The postulated degradation pathway is demonstrated in Fig. 6. The molecular oxygen adds onto the C-13 position and detaches a hydrogen from the allylic position (C-16) to form an allylic hydroperoxide (Schenck reaction) (Coxon and Halton, 1974). The reaction is characterized by double bond migration. It takes place by either the ene or the perepoxide mechanism. This allylic hydroperoxide intermediate then undergoes dehydration to yield MPP.

In conclusion, we have established that alkannin/shikonin is a light-sensitive compound in chloroform. The major photolytic product was identified to be (−)-5,8-dihydroxy-2-(1-hydroxy-3-oxo-4-methyl-4-pentenyl)-1,4-naphthoquinone, a new chemical entity. Its pharmacological effects and toxicity remain to be investigated. In light of this finding, precautions should be taken to avoid light exposure to ensure the stability of pharmaceutical products that contain alkannin/shikonin.

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