J. Korean Soc. Food Nutr.
24(3), 459~465(1995)

한국영양식량학회지

GC-MS Analysis of Diterpene Quinone Constituents of Salviae Miltiorrhizae Radix and Biological Activity

Hee-Juhn Park†, Seung-Bae Lee*, Eun Lee, Bae-Chun Cha**, Moo-Young Choi***, Sung Mok Lee**** and Won Tae Chung****

Dept. of Botanical Resources, Sangji University, Wonju 220-702, Korea
**Dept. of Dairy Food Science, Sangji University, Wonju 220-702, Korea*
***Dept. of Animal Nutritional Resources, Sangji University, Wonju 220-702, Korea*
****Dept. of Food Science & Nutrition, Sangji University, Wonju 220-702, Korea*
*****Central Research Institute, Il-Yang Pharm. Co., Yongin 449-900, Korea*

Abstract

The ether extract of Salviae miltiorrhizae Radix(SMR) was fractionated to give five subfractions, so that two subfractions of them were recrystallized to yield each pure diterpene quinone pigment. On the basis of spectral evidence, these two compounds were identified as tanshinone II and cryptotanshinone. Cryptotanshinone exhibited both of a potent platelet anti-aggregating activity *in vitro* and a potent antimicrobial activity. GC-MS analysis of the ether extract showed that tanshinone II was contained in the largest proportion of all the diterpene quinones. In addition, GC-MS analysis gave other valuable analytical informations.

Key words : *Salvia miltiorrhiza*, Labiate, cryptotanshinone, anti-platelet aggregation, antimicrobial, GC-MS

INTRODUCTION

Salvia miltiorrhiza Bge (Labiatae) is a perrenial herb, the root of which, Salviae miltiorhizae Radix (SMR), has been used for treatment of menstrual disorder, menostasis, insomnia, blood circulation disease and angina pectoris as well as against inflammation (1).

Since this crude drug is assumed to act on blood circulation system, recent researches on SMR (2) have mainly been focused on the development of platelet aggregating inhibitor like the example of paeonol isolated in Paeoniae Radix.

Chung *et al.* reported that magnesium lithospermate, hydrophilic lignan constituent, of this medicinal drug effectively improve renal failure (3) and exhibitantioxidant activity (4).

In addition, SMR contain diterpene quinone in relatively large amount. Among them, one of the diterpene quinone of SMR, tanshinone II, was prepared to the salt, tanshinone II sulfonate, so that it treats angina pectoris through the dilation of coronary arteries (1).

Lee *et al.* (5) reported that isotanshinone IIB of SMR showed platelet anti-aggregating activity. Many research workers have reported antibacterial activity and cytotoxic activity of diterpene quinone pigment of SMR (6). The constituents in SMR are classified into hydrophilic phenolic constituents such as salvianolic acid (7) as well as magnesium lithospermate and lipophilic diterpene quinone pigment (8).

Thus, the research on platelet anti-aggregating inhibitor was carried out in order to produce the an active extract. It was extracted with water and ether, repectively. In addition, for proving antimicrobial substance among SMR, activity guided fractionation on the ether extract was carried out. And also minimal inhibitory concentration (MIC) tests of an active principle were performed against several microorganisms.

This results of this study found that cryptotanshinone possess both potent inhibitory activity on platelet aggregation and on microbial growth.

Additional research is need because of the complexity of variable similar diterpene quinone and the an-

†To whom all correspondence should be addressed

alysis applicable to GC-MS is very profitable, this paper deals also with the analysis of diterpene quinone by GC-MS. While HPLC analysis (9) on abietane - *type pigment of SMR were reported, no GLC analysis* on them was suggested.

EXPERIMENTAL METHODS

All melting point were measured on an Electrothermal digital melting point apparatus and uncorrected. The IR spectra were determined in KBr tablets on a Bomem MB-100 FT-IR spectrometer and the UV spectra were run Shimadzu UV-160 UV/vis recording spectrophotometer.

The ^{1}H- and ^{13}C-NMR spectrum using TMS as an internal standard were measured. The EI mass spectra were taken with Finnigan Mat TSQ-700. For TLC, Kieselgel 60 F_{254} sheets (Merck) were used.

Plant material and extraction

Commercially available S. miltiorrhizae Radix were extracted with ether using Soxleht apparatus and followed by concentrated *in vacuo* to yield ether extract (32g).

The SMR, 600g, was extracted by hot extraction and further it was concentrate *in vacuo* to yield 89g of dry weight.

Isolation of compound 1 and 2 from ether extraction

Ether extract was subjected to SiO_2 column chromatography by the gradient development of n-hexane-EtOAc solvent system and was afforded subfraction 1~5. Among them, Fr. 4 anf Fr. 5 afforded compound 1 and compound 2, respectively, by recrystalization in n-hexane.

Compound 1 (tanshinone II)

mp 196~198° C, UV, λ_{max} (MeOH)nm (log ε) : 225 (4.31), 251 (4.30), 268 (4.46), 350 (3.22), 460 (3.47) ; IR, γmax (KBr)cm^{-1} : 1690, 1670 (carbonyl), 1580, 1535, 1460 (aromatic C=C), 1381, 1284, 1191, 1159 ; ^{1}H-NMR (200MHz, $CDCl_3$)δ : 1.32 (6H, s, C_6-geminal dimethyl), 1.72 (4H, m, $C_{7,8}$-H), 2.27 (3H, d, J= 2Hz, C_1-CH_3), 3.20 (2H, t, J=6.42 Hz, C_9), 7.22 (1H, s, C_2-H), 7.56 (1H, d, J=8.1 Hz, C_3-H), 7.64 (1H, d, J=8.1 Hz, C_4-H) ; ^{13}C-NMR (50MHz, $CDCl_3$)δ : 8.7 (C_1-CH_3), 19.8 (C_7), 30.0 (C_9), 32.1 (C_6-CH_3), 31.7 (C_6-CH_3), 37.8 (C_7), 119.8 (C_1), 121.1 (C_{11a}), 126.4 (C_{9b}), 127.4 (C_{3b}), 125.2 (C_4), 133.4 (C_5), 141.2 - (C_2), 150.1 (C_{9a}), 177.2 (C_{10a}), 185.0 (C_{10}), 34.6 (C_6), 19.1 (C_8) ; MS, m/z (%) : 294 $[M]^+$, 279 $[M-CH_3]^+$, 251 $[M-CH_3-CO]^+$(42), 233 $[M-CH_3-CO-H_2O]^+$(17), 178 (b_1,18), 165 (c_1, 23), 152 (d_1, 21)

Compound 2(cryptotanshinone)

mp 191~192° C, UV, λ_{max} (MeOH)nm (log ε) : 263 (4.46), 271 (4.41), 355 (3.41), 477 (3.48) ; IR γ_{max} (KBr)cm^{-1} : 2950 (CH), 1680, 1648 (carbonyl), 1620, 1553, 1460 (aromatic C=C), 1400, 1333, 1193, 1140, 840, 700 ; ^{1}H-NMR (200MHz, $CDCl_3$)δ : 1.38 (6H, s, C_6-geminal dimethyl), 1.45 (3H, d, J=6Hz, C_1-CH_3), 1.80 (4H, m, $C_{7,8}$-H), 3.65 (1H, m, C_1-H_x), 4.37 (1H, dd, J_{AB}=Hz, J_{AX}=5Hz, C_2-Ha), 4.92 (1H, t, J_{AB}=J_{BX}=9 Hz, C_2-H_B), 7.48 (1H, d, J=8.5 Hz, H-5), 7.62 (1H, d, J=8.5 Hz, H-4) ; ^{13}C-NMR (50MHz, $CDCl_3$)δ : 18.0 (C_1-CH_3), 18.3 (C_8), 40.0 (C_1), 80.8 (C_2), 170.2 (C_{3a}), 127.5 (C_{3b}), 121.9 (C_4), 132.0 (C_5), 142.7 (C_{5a}), 34.1 (C_6), 31.2 (C_6-CH_3), 33.8 (C_6-CH_3), 37.0 (C_7), 29.0 (C_9), 151.6 (C_{9a}), 125.5 (C_{9b}), 183.5 (C_{10}), 174.8 (C_{10a}), 117.5 (C_{11a}) ; MS m/z (%) : 296 $[M]^+$, 281 $[M-CH_3]^+$, 253 $[M-CH_3-CO]^+$, 235 $[M-CH_3-CO-H_2O]$+, 179 (a_1, 41), 141 (b_2, 63), 128 (c_2, 84), 115 (d_2, 97)

GC-MS analysis of ether extract

The diterpene quinone of ether extract was analysed by GC-MS apparatus (Instrument : HP 5890 series II gas chromatography-HP 5970 mass selective detector. Condition ; column : ultra-2 (25m × 0.2 mm. i.d., 0.33μm *thickness, oven temp.* -150° C *for 2 minutes* and then was raised to 280° C, injector temp. -280° C, *interface temp.* -300° C, *ionization mode-EI mode).* Total ion chromatogram was shown in Fig. 1.

The experiment on inhibitory activity against collagen induced-platelet aggregation

The blood of rabbits anesthetized with pentobarbital was gathered by the syringe filled with 3.2%-trisodium citrate. Platelet-rich plasma (PRP) was obtained by the centrifuge of this blood for 10 minutes (150g).

And further platelet-poor plasma (PPP) was obtained by centrifugation of the precipitate of PRP at 1500g for 10 minutes. The measurement of platelet aggregation was carried out by the turbidometric method using aggregometer. After PRP (351 μl where 5%-DMSO (vehicle, 25 μl) or test substances (25 μl) was added and followed by stirring and incubating at 37° C, platelet aggregation was induced by collagen.

Antimicrobial activity test

To culture the microorganisms, nutrient agar and nutrient broth (DIFCO Laboratories) were used. Before testing, the microorganisms were cultured in the liquid nutrient broth at 30° C overnight. For the paper disc test, about 0.5ml sample of the precultured microorganisms cell broth was taken and smeard uniformly on an agar plate made of 20ml of nutrient agar.

Paper discs (Toyo Roshi Co., 8mm dia. and about 1.5mm thick) containing 1mg/ml fractions obtained from the chromatographic fractionation and the same amount of isolated compound were placed on the seeded plates.

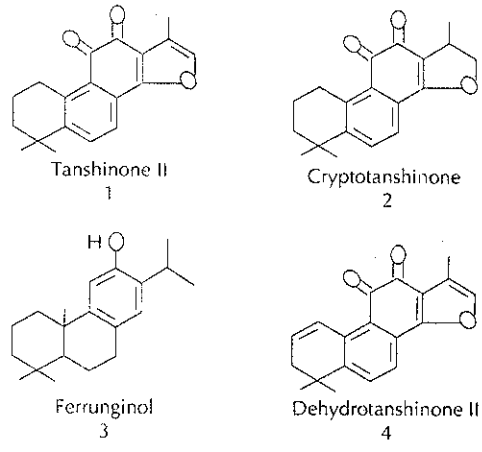
They are incubated at 30° C for 48 hours, so that growth inhibitory zones were visually compared.

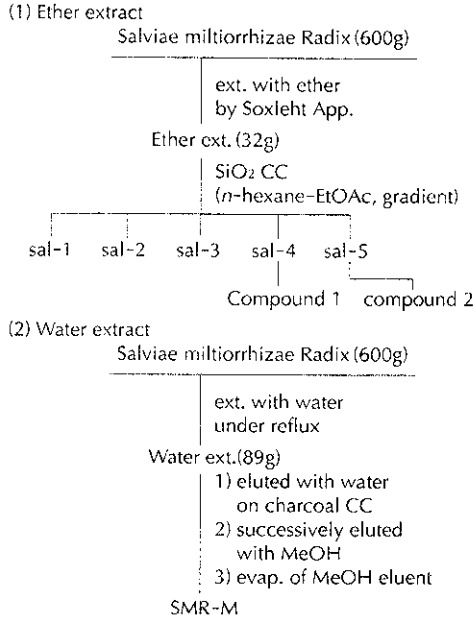
To measure the minimal inhibitory concentration, the five samples were examined against several microorganisms by serial dilution as follows : 1ml of the solution to be tested was added to 9ml of sterile media containing 1.5% agar at around 50° C in the petri dish. This mixture was shaken thoroughly and solidified. One platinum loop of the organisms suspension precultured at 30° C overnight was inoculated on the agar medium, and incubated at 30° C for 48 hours. The minimal inhibitory concentration was estimated by visually comparing the microorganisms growth.

RESULTS AND DISCUSSION

The methanolic extract was chromatographed to give five subfraction from sal-1 to sal-5 as shown in Scheme 1. Two subfractions, sal-4 and sal-5, among them were recrystallized with each n-hexane to afford compound 1 and 2, respectively as shown in Scheme 1.

Because spectral data of UV, IR, ^{1}H-NMR and ^{13}C-





Scheme 1. Extraction and isolation procedures of Salviae miltiorrhizae Radix.

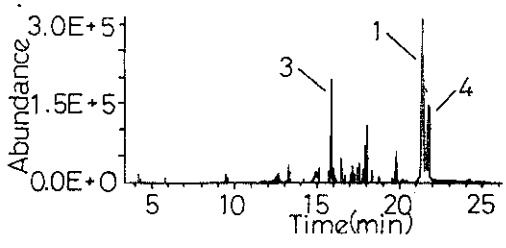


Fig. 1. Total ion chromatogram of SMR ether extract by GC-MS.

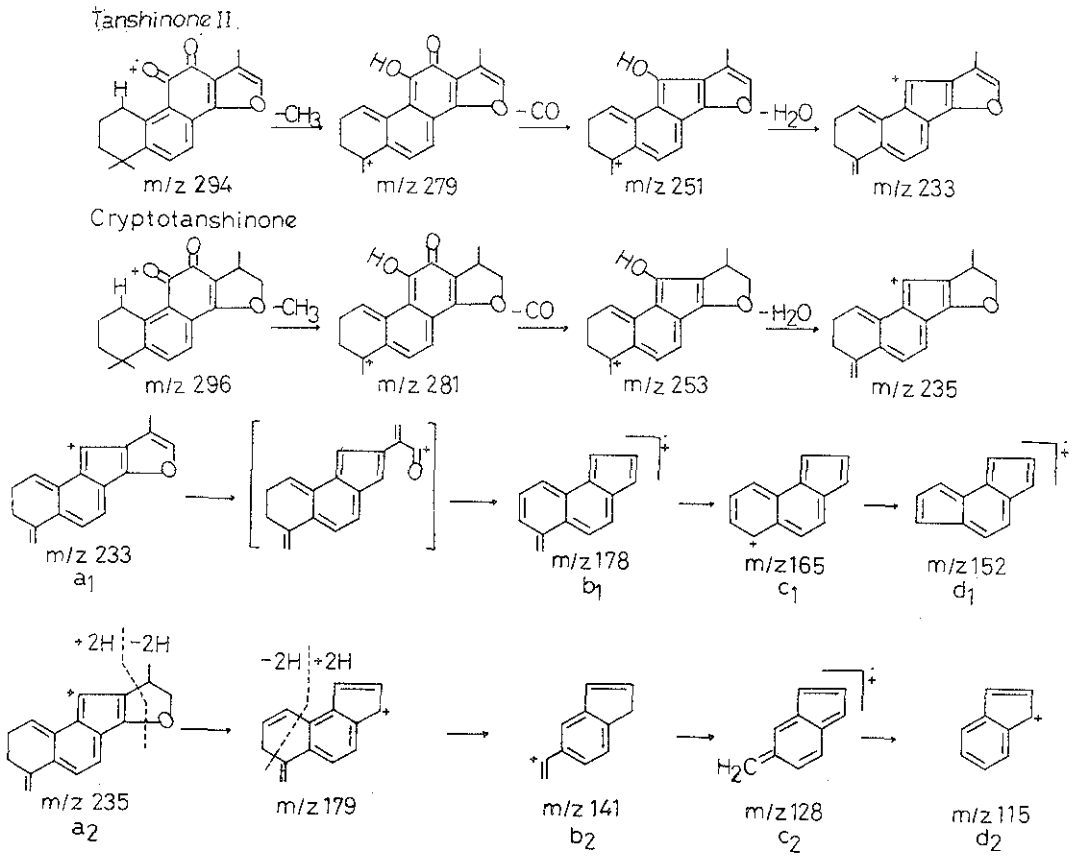


Fig. 2. Possible mass fragmentation of tanshinone II and cryptotanshinone.

NMR of compound were in accordance with those of tanshinone II as already reported in the literature, it was confirmed as tanshinone II. In the same way, compound 2 was identified to be cryptotanshinone by the comparison with those reported in the literature. These two compounds have already been known from SMR.

From the mass fragmentation of compound 1 and 2 was diterpene quinone analogues obtained from Salviae miltiorrhizae Radix.

It was speculated that Salviae militiarrhizae Radix was produced because of McLafferty rearrangement and successive elimination of methyl, carbonyl and H_2O as shown in Fig. 2.

Since many of these analogues have furan or dihydrofuran at D-ring, it is important to interpretate low fragment ion in order to identify partial structure at D-ring, i.e., the prominent peaks such as m/z 178 (b_1), m/z 149 (c_1) and m/z 152 (d_1) means the presence of furan moiey at D-ring and also the prominent ion peak like m/z 141 (b_2), m/z 128 (c_2) and m/z 115 (d_2) means the presence of dihydrofuran ring at D-ring site of the skeleton (Fig. 1).

Since diterpene-1,2-quinone possessing abietane structure of SMR include other substituted group only at A- or D-ring, it is very profitable to examine this fragmentation pattern. Because of this convenient interpretation from mass spectrum, GC-MS analysis on total ether extract was carried out. As a result, we obtained total ion chromatogram (Fig. 3) and each corresponding mass spectrum (Fig. 4).

As shown in Fig. 4, compound 1 was identified as tanshinone II by the comparison of mass fragmentation with that of tanshinone II (1).

Highest peak of 1 in this chromatogram indicated that this compound was contained in the largest amo-

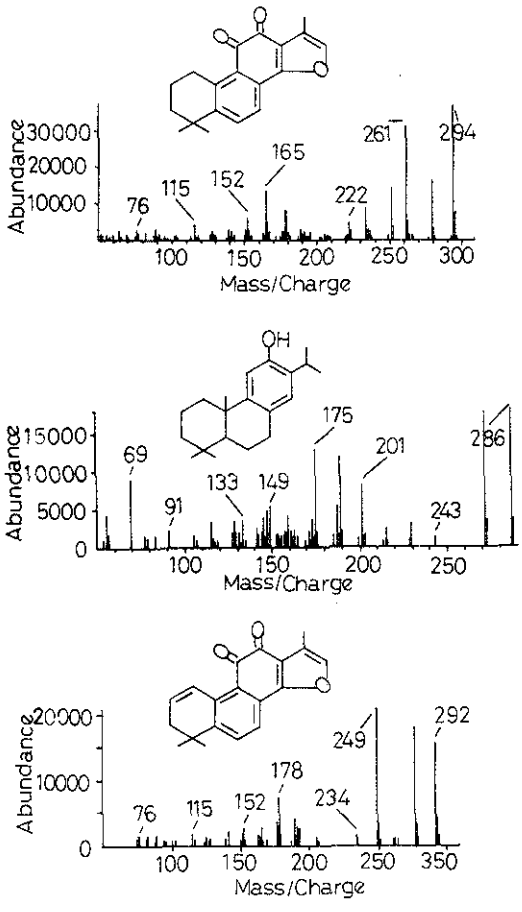


Fig. 3. MS spectra of tanshinone II (1), ferrunginol (3) and dehydrotanshinone II (4) from GC-MS.

unt of diterpene quinone.

From mass fragmentation shown in Fig. 4 compound 3 was identified as ferrunginol. No occurrence of H_2O elimination from the structure possessing phenolic OH before cleaving the benzene moiety and the presence of isopropyl group at the D-ring, instead of furan moiety recognized from the molecular ion make it concluded that it was ferrunginol (Fig. 5).

Successive elimination of methyl and carbonyl from compound 4 occurred, it produced m/z 277 and m/z 249 (Fig. 5).

Although it was assumed as 6,7-dihydrophenanthro[1,2-b]-furan-10,11-dione from the examination of the fact that 9-hydroxytanshinone II was present in SMR, the possibility of the presence of olefin in C-7

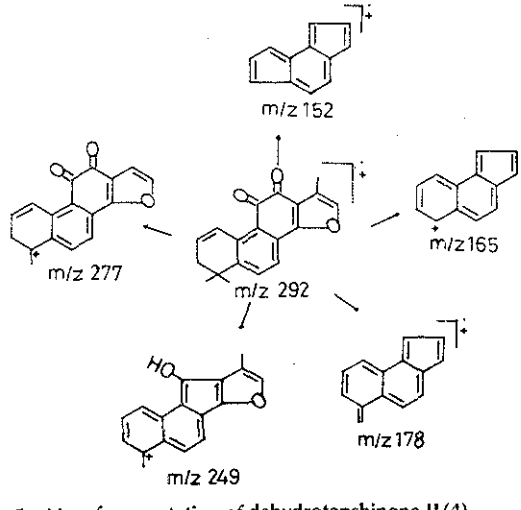


Fig. 5. Mass fragmentation of dehydrotanshinone II (4).

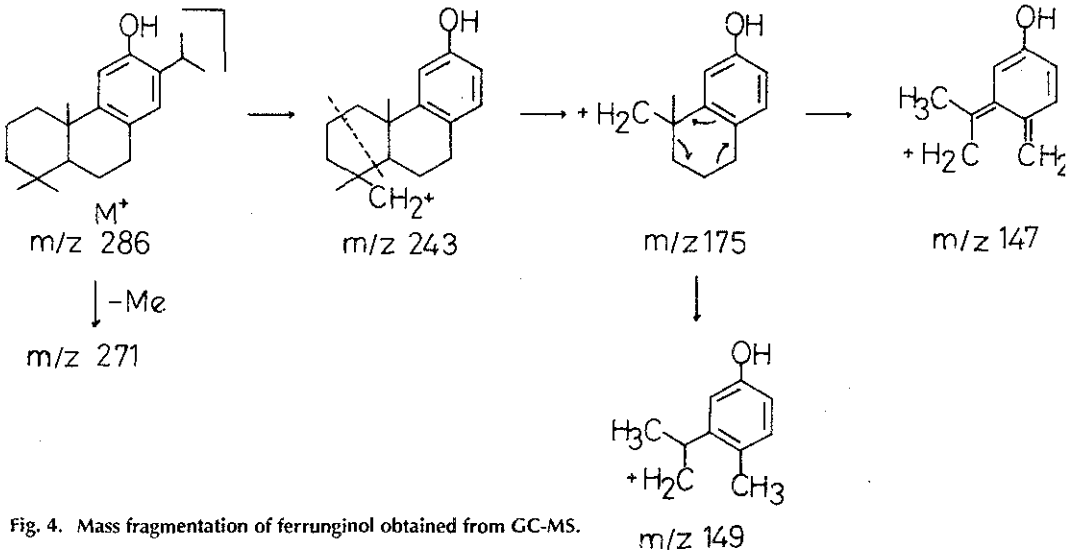


Fig. 4. Mass fragmentation of ferrunginol obtained from GC-MS.

was not excluded.

As shown in Table 1, collagen induced platelet anti-aggregating activity test *in vitro* revealed that cryptotanshinone from SMR exhibited the activity superior to paeonol of naturally occurring platelet aggregating inhibitor in the weight per volume concentration. As the IC_{50} level of activity of cryptotanshinone was similar to that of 0.250mM of aspirin, it was suggested that this compound was considerably strong platelet aggregating inhibitor.

However, even the high concentration (1,000μg/ml) of water extract of SMR did not exert this action.

Therefore, it was suggested that the effective substances improving blood circulating activity was involved in diterpene quinone portion containing cryptotanshinone and *etc.*

The microorganisms tested in the present MIC tests on the antimicrobial activity was shown in Table 2.

Sal-1 was not active at all against all the tested microorgnisms. While sal-2, sal-3 and tanshinone II were active against only a few species, cryptotanshinone showed potent antimicrobial activities against all the tested microorganisms as shown in Table 2.

As measured MIC values of cryptotanshinone was shown in Table 3, this compound completely inhibited the gowth of *Sacchromyces cerevisiae, Candida albicans, Streptococcus mutans, Bacillus licheniformis* and *Pseudomonas aeruginosa* in 12.5μg/ml. By contrast, *Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis* and *Saccharomyces rouxii* inhibited the growth in 500, 200, 25 and 25μl/ml respectively.

Table 1. Effect of test compound on the platelet aggregation by collagen (10μg/ml)

Treatment	Concentration	% of inhibition
Cryptotan shinone	200μg/ml	48.7
	50μg/ml	41.4
Paeonol	100μg/ml	21.3
Aspirin	0.500mM	85.0
	0.250mM	45.4
	0.125mM	22.3
Water ext. of SMR	1,000μg/ml	0.0

CONCLUSIONS

Since the biologically active substance among SMR

Table 3. Minimal inhibitory concentrations of cryptotanshinone on microbial growth

Organisms	MIC (μg/ml)
Streptococcus mutans KDC 3065	12.5
Lactobacillus casei IFO 12004	500
Pseudomonas aeruginosa IFO 3899	12.5
Bacillus licheniformis ATCC 21415	12.5
Bacillus subtilis ATCC 6633	25
Bacillus stearothermophilus KFCC 11238	12.5
Klebsiella pneumoniae IFO 3321	500
Staphylococcus aureus IFO 12732	200
Escherichia coli ATCC 15491	1000
Enterobacter aerogenes ATCC 29751	100
Sacharomyces rouxii	25
Saccharomyces cerevisiae IFO 1346	12.5
Candida albicans ATCC 10231	12.5
Saccharomyces formonsensis	1000

Table 2. Antimicrobial activity of sal-1, sal-2, sal-3, tanshinone II and cryptotanshinone on microbial growth

Organisms	Clear zone of plate (mm)				
	Sal-1	Sal-2	Sal-3	Tanshinone II	Cryptotanshinone
Streptococcus mutans KDC 3065	–	–	–	10	13
Lactobacillus casei IFO 12004	–	–	–	–	13
Pseudomonas aeruginosa IFO 3899	–	–	–	–	23
Bacillus licheniformis ATCC 21415	–	–	11	12	16
Bacillus subtilis ATCC 6633	–	–	–	–	12
Bacillus stearothermophilus KFCC 11238	–	11	–	14	22
Klebsiella pneumoniae IFO 3321	–	10	–	–	14
Staphylococcus aureus IFO 12732	–	12	14	–	12
Escherichia coli ATCC 15491	–	10	–	–	14
Enterobacter aerogenes ATCC 29751	–	–	–	–	12
Sacharomyces rouxii	–	–	–	–	15
Saccharomyces cerevisiae IFO 1346	–	–	–	–	12
Candida albicans ATCC 10231	–	–	–	–	13
Saccharomyces formonsensis	–	–	–	–	12

was occurred in the group of phenolic compound and the group of diterpene quinone compound, the present study deals with the distribution of platelet aggregating inhibitor in SMR. In addition, diterpene quinones exhibiting the complex composition was analyzed by GC-MS.

The occurrence of McLafferty rearrangement and prominent fragment ion peak in mass spectrum make it profitable to determine the chemical structure.

Compound 4 showed in GC-MS was assumed to be 6,7-dihydrophenanthro[1,2]-furan-10,11-dione through the examination of molecular ion peak and its mass fragmentation procedures.

In the blood circulating activity based on platelet anti-aggregating test, cryptotanshinone revealed that it was very important platelet aggregating inhibitor which IC_{50} of this compound with on activity level in 0.250mM of aspirin. Therefore, it was deduced that platelet aggregating inhibitor was mainly involved in lipophilic portion such as diterpene quinone.

Moreover, since cryptotanshinone exhibited antimicrobial activity against several species of bacteria and yeast in the low concentration (12.5μg/ml) in addition to platelet anti-aggregating activity, it was deduced that it was available biologically active substance.

REFERENCES

1. Tang, W. and Eisenbrand, G. : Chinese drugs of plant origin. Springer-Verlag, Berlin, p.891 (1992)
2. Wang, N., Luo, H. W., Niwa, M. and Ji, J. : A new platelet aggregation inhibitor from *Salvia miltiorrhiza*. *Planta Med.*, **55**, 390 (1989)
3. Chung, H. Y., Yokozawa, T., Lee, T. W., Oura, H., Tanaka, T., Nonaka, G. and Nishioka, I. : Effect of magnesium lithospermate B on urinary excretion of arachidonate metabolites in rats with renal failure. *Chem. Pharm. Bull.*, **37**, 2766 (1989)
4. Paik, K. J. : Studies on the antioxidative mechanisms and active sites of magnesium lithospermate B. *M.S. Thesis*, Pusan National University (1994)
5. Lee, A. R., Wu, W. L., Chang, W. L., Lin, H. C. and King, M. L. : Isolation of bioactivity of new tanshinones. *J. Nat. Prod.*, **50**, 157 (1987)
6. Ulubelen, A., Evren, N., Tuzlaci, E. and Johansson, C. : Diterpenoids from the roots of *Salvia hypargeia*. *J. Nat. Prod.*, **51**, 1178 (1988)
7. Li, L., Tan, R. and Chen, W. : Salvianolic acid A, a new depside from roots of *Salvia miltiorrhiza*. *Planta Med.*, **15**, 227 (1984)
8. Onitsuka, M., Fufiu, M., Shinmka, N. and Maruyama, H. B. : New platelet aggregation inhibitors from Tanshen : Radix of *Salvia miltiorrhiza* Bunge. *Chem. Pharm. Bull.*, **31**, 1670 (1983)
9. Okamura, N., Sato, M., Yagi, A., Tanonaka, K. and Tkeo, S. : An application of HPLC for identification of abietane-type pigment from *Salvia miltiorrhiza* and their effects on post-hypoxic cardiac contractile foerce in rats. *Planta Med.*, **58**, 571 (1992)

(Received April 26, 1995)

단삼의 Diterpene Quinone 성분의 GC-MS에 의한 분석 및 생리활성

박희준† · 이승배* · 이 은** · 차배천** · 최무영*** · 이승목**** · 정원태****

상지대학교 자원식물학과

*상지대학교 낙농식품자원학과, **상지대학교 동물영양자원학과

***상지대학교 식품영양학과, ****일양약품 중앙연구소

요 약

단삼의 혈소판응집 억제 활성물질이 지용부 및 수용부에 분포한다는 각각 다른 보고가 있으나 본 연구에서는 수용부는 혈소판응집억제 활성을 나타내지 않았으며 diterpene quinone 성분의 하나인 cryptotanshinone은 현저한 혈소판응집억제 활성을 나타내었으므로 이의 유효생리활성물질은 diterpene quinone 색소에 분포할 것으로 추측된다. 뿐만 아니라, cryptotanshinone은 항균작용도 동시에 나타내는 생리활성물질이었다. 또한, 복잡한 조성을 나타내는 단삼의 지용성 색소 성분의 분석을 위해서 GC-MS 분석을 행한 결과 유용한 분석적 결과를 제시하였다. 즉, 가장 많은 색소 성분은 tanshinone II이었으며, 또한 새로운 diterpene quinone 성분의 존재가 시사되었다.