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Detection of free radical scavenging activity of schisanhenol by electron spin resonance

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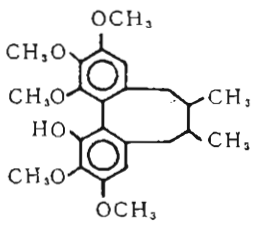
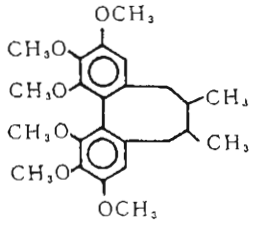
ABSTRACT It was reported that Schisanhenol (Sal) isolated from *Schisandrae Rubriflora* inhibited lipid peroxidation induced by ferrous–cysteine and NADPH–ascorbic acid. In this studies the oxygen radical scavenging activity of Sal was detected by electron spin resonance (ESR) and spin trapping. Sal was shown to scavenge oxygen radicals produced by human neutrophils (Neu) stimulated by tetradecanoylphorbol acetate (TDPA). But no effect of Sal was seen on oxygen consumption measured by spin label oximetry in Neu during respiratory burst. In Fenton reaction system, the inhibitory rate of hydroxyl radical by Sal was 34.4% . In xanthine–xanthine oxidase and uv–irradiation of riboflavin systems, Sal scavenged superoxide anion radical by 26.1% and 21.9% , respectively. In all these systems the action of Sal was more potent than that of vitamin E. It may be concluded that Sal possesses a free radical–scavenging activity.

KEY WORDS schisanhenol; schisandrin A; electron spin resonance; free radicals; neutrophils

Fructus schisandrae has been used for centuries as astringent and tonic in traditional Chinese medicine. Recently, it was found that certain dibenzocyclooctene lignans isolated from the seeds of this plant inhibited CCl_4 induced liver injury[1], CCl_4[2], ferrous–cysteine and NADPH ascorbic acid[3] induced lipid peroxidation of liver microsomes as well as H_2O_2 induced hemolysis and lipid peroxidation of rat erythrocytes. These results indicated that the lignans have antioxidant activity. Of these lignans the most potent one, Schisanhenol (Sal) and a less potent one, Schizandrin A (Sin A), were selected for evaluation of their free radical–scavenging activity by electron spin resonance (ESR).



Schisanhenol (Sal)



Schisandrin A (Sin A)

MATERIALS AND METHODS

Sal and Sin A were obtained from the Department of Phytochemistry of the Institute of Materia Medica, Chinese Academy of Medical Sciences. Both compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffer solution (PBS), 0.05 mol / L, pH 7.4, before use. 5,5–Dimethyl–1–pyrroline–1–oxide (DMPO) purchased from Sigma was purified with active charcoal till no signals of impurity were found on ESR spectra. 3–Carbamoyl–2,2,5,5–tetramethyl–3–pyrroline–1–yloxy (CTPO) from Sigma was dissolved in a small amount of ethanol and diluted with PBS 0.05 mol / L (pH 7.4) before use. Tetradecanoylphorbol acetate (TDPA, Sigma) was dissolved in an adequate amount of acetone and stored at – 20 ℃ till use. It was diluted with PBS 0.05 mol / L (pH 7.4) before use. Hydrogen peroxide 30% (product of Beijing Chemical Agent Manufactory) was diluted

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with ion free water before use. Xanthine (Sigma) was dissolved and diluted with PBS 0.05 mol / L (pH 8.0). Xanthine oxidase purchased from the Shanghai Institute of Biochemistry, Chinese Academy of Sciences. Riboflavin, a product of Shanghai Chemical Agent Manufactory, was dissolved and diluted with PBS 0.05 mol / L (pH 5.0). All chemicals used are of analytical grade.

Active oxygen radicals produced by Neu Fresh whole blood of healthy donor (ACD–B) was obtained from Beijing Red Cross. Neu were seperated and purified by centrifugation in dextran 70[9]. A mixture of 40 μl containing Neu 1×10^7cells / ml, diethylene triamine pentacetic acid (DETAPAC) 0.1 mmol / L, TDPA 100 ng / ml and Sal or Sin A (1 mmol / L) was incubated for 2 min at 37 ℃, then 0.1 mol / L DMPO was added. The reaction mixture was immediately transferred to a quartz capillary and fitted into the cavity of the ESR spectrometer (Varian E–109). Microwave power was 15 mW, X band, modulation frequency was 100 kHz with an amplitude of 1.0 G, scaning time was 50 G / min and the receiver gain was 2.5×10^4 with a time constant of 0.128. The temperatur was 25 ℃

Hydroxyl free radical ($OH^{\cdot}$) produced in Fenton reaction system A mixture of 20 μl containing H_2O_2 1%, ferrous ammonium sulfate 100 mol / L and DMPO 0.1 mol / L were mixed (pH 7.4). The hydroxyl radical spin adducts of DMPO (DMPO–OH) were detected[5].

Superoxide anion ($O_2^{\cdot}$) produced in xanthine–xanthine oxidase system A mixture of 20 μl (pH 8.0) containing xanthine 0.4 mmol / L, DETAPAC 0.1 mmol / L, xanthine oxidase 0.1 IU / ml and DMPO 0.1 mol / L was prepared. The $O_2^{\cdot}$ spin adducts of DMPO (DMPO–OOH) were detected immediately as described above.

$O_2^{\cdot}$ produced in uv–irradiation of riboflavin system A mixture (pH 5.0) of 20 μl containing riboflavin 0.3 mmol / L, EDTA 5 mmol / L and DMPO 0.1 mmol / L was transferred into a quartz capicillary and put into the cavity of ESR spectrometer. After irradiation of the sample for 30 s with xenon lamp (power 1 kW, distance 70 cm), DMPO–OOH were detected immediately. ESR measurement conditions were the same as described above.

Measurement of oxygen consumption during respiratory burst in Neu Measurement equation[4,7] was : $K=(b+c)/2a$. a, b, c, were shown in Fig 2. ESR measurement conditions were: microwave power, 1 mW; X band; modulation frequency, 100 kHz; amplitude, 0.005 G; scanning time, 0.63 G / min and time constant, 0.128. The temperature was 25 ℃.

RESULTS

Active oxygen radical–scavenging effects of Sal and Sin A in Neu during respiratory burst Neutrophils (1×10^7 cells / ml) in DETAPAC 0.1 mmol / L and DMPO 0.1 mol / L were stimulated with TDPA (100 ng / ml) and examined by ESR (Fig 1). The resulting spectrum was a composite one of 2

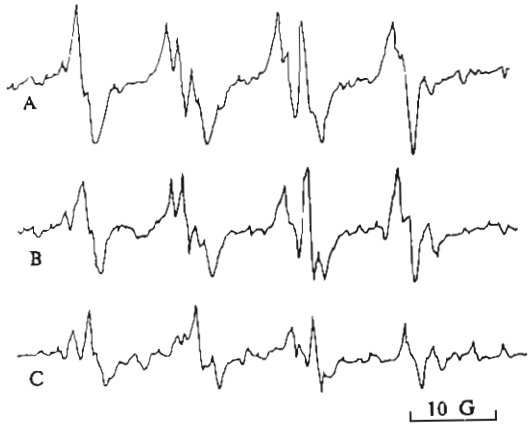


Fig 1. Effects of schisanhenol (Sal) and schisandrin A (Sin A) on $O_2^{\cdot}$ and $OH^{\cdot}$ production in tetradecanoylphorbol (TDPA) stimulated human neutrophils (Neu) *in vitro*. A) Control B) Sin A 1 mmol / L. C) Sal 1 mmol / L.

distinct products: the superoxide anion adduct (DMPO–OOH) and hydroxyl adduct (DMPO OH). The size of the DMPO–OOH and DMPO–OH peaks was decreased by Sal (Fig 1C) and Sin A (Fig 1B). This result showed that Sal and Sin A scavenged active oxygen radicals produced by Neu during respiratory burst. Sal was more potent than Sin A.

Effects of Sal and Sin A on oxygen consumption of Neu during respiratory burst stimulated by TDPA In order to exclude the possibility that Sal and Sin A reduced oxygen radical production by Neu through inhibiting oxygen consumption during respiratory burst, spin label oximetry was used to determine *K* value (Fig 2) for evaluating the effect of Sal and Sin A on oxygen consumption of Neu stimulatelted by TDPA. As shown in Fig 3, Sal and Sin A did not affect *K* value. It indicated that Sal and Sin A had no effect on oxygen consumption of Neu during respiratory burst stimulated by TDPA.

Sal and Sin A scavenging OH˙ in Fenton reaction OH˙ was generated according to Fenton reaction:

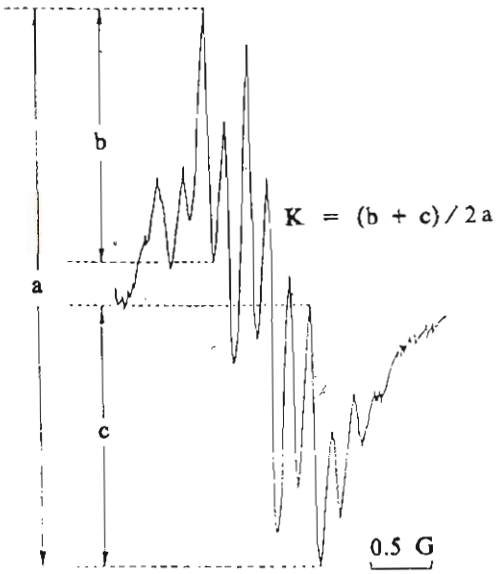


Fig 2. Superhyperfine structure of the electron spin resonance spectrum of nitroxide 3–carbamoyl–2,2,5,5–tetramethyl–3–pyrroline–1–yloxy (CTPO) showing the definition of *K* parameter.

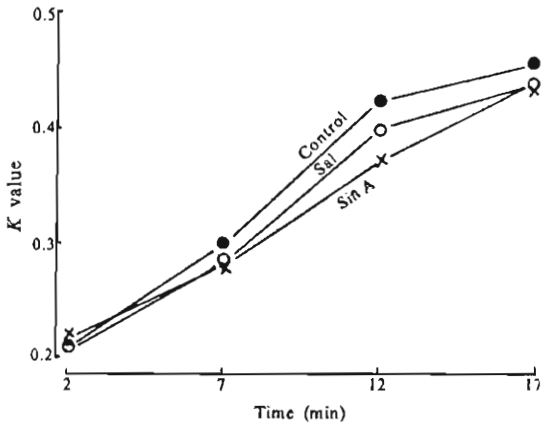


Fig 3. Effects of Sal (●) 1 mmol / L and Sin A (×) 1 mmol / L on oxygen consumption of TDPA stimulated human neutrophils *in vitro*. (O) Control.

$$Fe^{2} + H_2O_2^{\cdot} \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

The hydroxyl spin adduct spectra of DMPO produced by a solution of DMPO 0.1 mol / L, 1% H_2O_2 and ferrous ammonium sulfate 100 μmol / L were obtained. The scavenging rate (E) was defined as : $E = ((h_0 - h_x) / h_0) \times 100\%$. Here h_0 was the height of the second peak of control sample in ESR spectra in Fig 4A and the 1st peak in Fig 4 B and Fig 4C, h_x was the height of the 2nd peak in Fig 4A and the 1st peak in Fig 4B and Fig 4C after Sal, Sin A, Vit E or ascorbic acid were added. Hydroxyl radical–scavenging activity of both Sal and Sin A was stronger than that of Vit E and weaker than that of ascorbic acid (Tab 1). Hydroxyl radical–scavenging activity of Sal was stronger than that of Sin A ($P < 0.05$)

Sal and Sin A scavenging $O_2^{\cdot}$ in xanthine–xanthine oxidase system Fig 1 was a composite drawing of DMPO–OH and DMPO–OOH. In order to evaluate the superoxide anion–scavenging activity of Sal and Sin A, xanthine–xanthine oxidase system was used for producing $O_2^{\cdot}$, but not OH˙. The superoxide spin adduct spectrum of DMPO produced by a solution of xanthine 0.4 mmol / L, DETAPAC 0.1 mmol / L DMPO

0.1 mol / L and xanthine oxidase 0.1 IU / ml was obtained at pH 8.0, 1.5 min after the addition of xanthine oxidase (Fig 4B). The superoxide anion-scavenging activity of Sal was significantly stronger than that of Sin A ($P<0.01$) and similar to that of Vit E ($P>0.05$) (Tab 1). The superoxide anion-scavenging activity of both Sal and Sin A was weaker than that of ascorbic acid.

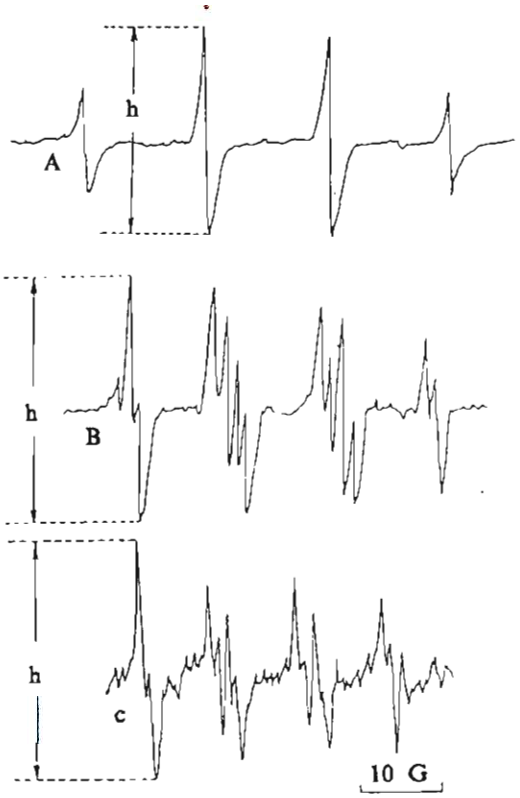


Fig 4. The hydroxyl radical ($OH^{\cdot}$) and superoxide anion ($O_2^{\cdot}$) spin adduct spectra of 5,5-dimethyl-1-pyrroline-oxide (DMPO). A) $OH^{\cdot}$ spin adduct in Fenton reaction system. B) $O_2^{\cdot}$ spin adduct in xanthine-xanthine oxidase system. C) $O_2^{\cdot}$ spin adduct in uv-irradiation of riboflavin system.

Sal and Sin A scavenging $O_2^{\cdot}$ in uv-irradiation of riboflavin system The superoxide anion spin adduct spectrum of DMPO produced by a solution of riboflavin 0.3 mmol / L, EDTA 5 mmol / L and DMPO 0.1 mol / L was obtained after uv-irradiation of the sample for 30 s with xenon lamp (Fig 4C). Superoxide anion-scavenging activity of both Sal and Sin A was essentially the same and equivalent to that of Vit E, but much weaker than that of ascorbic acid (Tab 1).

Tab 1. Scavenging rate of oxygen radicals (%) by Sal and Sin A. $n=5$ determinations, ($\bar{x}$ ± SD). All $P<0.05$ *vs* controls.

Drug (1 mmol / L)	Scavenging rate (%) Fenton reaction system	xanthine-xanthine oxidase system	Riboflavin system
Sal	38.8 ± 12.6	26.1 ± 3.7	21.9 ± 9.3
Sin A	25.5 ± 2.9	9.4 ± 3.5	24.8 ± 6.8
Vit E	21.0 ± 1.4	11.3 ± 3.1	22.8 ± 0.9
Ascorbic acid	62.2 ± 3.1	100 ± 0	100 ± 0

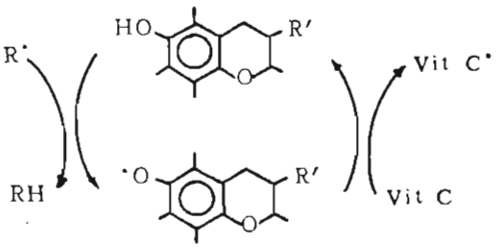
DISCUSSION

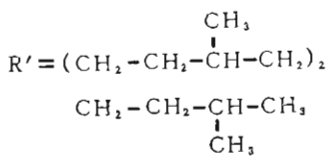
It has been confirmed that several compounds isolated from *Schisandrae* have antioxidant activities *in vivo* and *in vitro*[1-4]. Sal showed the strongest antioxidant activity[3]. It was speculated that they produced antioxidant effects by 1) inhibiting the production of free radicals; 2) scavenging free radicals; 3) increasing the activities of the antioxidant enzymes, superoxide dismutase and catalase. The results of the present experiment showed that Sal and Sin A had no effect on oxygen consumption of Neu during respiratory burst, while both of them decreased the size of the peaks of DMPO-OH and DMPO-OOH on ESR spectrum produced by Neu stimulated by TDPA during respiratory burst. It may be concluded that Sal and Sin A decreased the size of DMPO-OH and DMPO-OOH peaks by scavenging active oxygen radicals directly rather than inhibiting the production of oxygen radicals. It was further confirmed in Fenton reaction, xanthine-xanthine oxidase and uv-irradiation of riboflavin systems that Sal and Sin A could scavenge active oxygen radicals. Therefore, it appears that free radical-scavenging effect is one of the antioxidant

mechanisms of Sal and Sin A.

As shown in Fig 1 and Tab 1, the general tendency showed that active oxygen radical-scavenging activity of Sal was stronger than that of Sin A in three active oxygen radical-generating systems. These results were in accordance with the previous finding that Sal had a stronger antioxidant activity than that of Sin A[1-3]. The only difference in chemical structure between Sal and Sin A is that there is a phenolic hydroxyl group (-OH) in Sal and a $-OCH_3$ in Sin A. Therefore the hydroxyl group in Sal may be considered as one of the functional groups, responsible for more effective activity in scavenging active oxygen radicals.

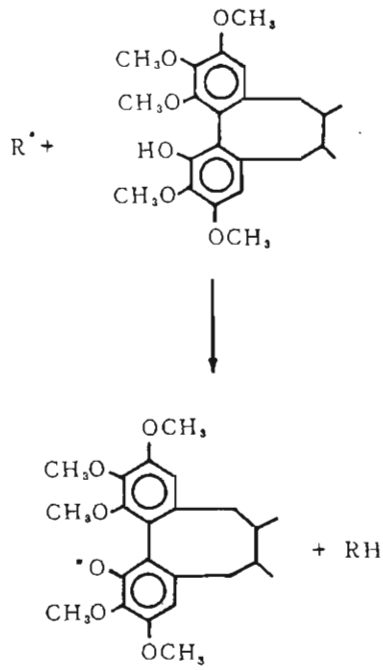
Vit E is a potent lipid soluble antioxidant. It scavenged free radicals in the following way[8]:





There is a similar phenolic hydroxyl group in the chemical structure of Sal and Vit E. The phenolic hydroxyl group of Vit E take part in scavenging free radicals, so it is possible that Sal scavenges free radicals in a way similar to that of Vit E, as shown below:

The mechanism of free radical scavenging effect of Sin A is unknown. There is possibility that the methoxyl group in the structure of Sin A is demethylated to form a hydroxyl group which is important for eleminating active oxygen species like Sal.



Finally, it should be mentioned that the free radical-generating systems used in the present experiment were water soluble. This may be the cause why the lipid soluble Sal, Sin A and Vit E showed weaker oxygen radical-scavenging activity than the water soluble ascorbic acid in these systems. Investigation of the oxygen radical-scavenging activity of Sal and Sin A in the lipid soluble system is of necessory.

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用电子自旋共振方法检测五味子酚清除自由基活性

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提要 从五味子中提取出的五味子酚(Sal)具有抗脂过氧化作用. 本文用电子自旋共振 electron spin resonance (ESR) 和自旋捕捉技术研究了 Sal 清除自由基的活性. 结果表明: Sal 清除由 tetradecanoylphorbol acetate (TDPA) 刺激人多形核白细胞(Neu)所产生的氧自由基. 自旋探针测氧法结果表明对 TDPA 刺激 Neu 时的氧消耗无影响. 在 Fenton 反应体系中, 对羟自由基的清除率为 34.4%, 在黄嘌呤–黄嘌呤氧化酶和光照核黄素体系中, 对超氧阴离子的清除作用分别为 26.1%和 21.9%. 在这些体系中, Sal 的作用均比维生素 E 强. 结果提示 Sal 具有直接捕获氧自由基的活性.

关键词 五味子酚; 五味子甲素; 电子自旋共振; 自由基; 嗜中性白细胞

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小檗胺的抗氧化作用

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Antioxidant effect of berbamine

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ABSTRACT The antiperoxidant action of berbamine (Ber) was demonstrated by colorimetric estimation of malondidehyde (MDA) formation, and the scavenging effect of Ber on O_2^- was investigated by chemiluminescence method and ESR–spin trapping technique. Ber 11–100 μmol / L remarkably inhibited MDA formation induced by incubating mice liver homogenate at 37℃ with vibration for 1 h. Ber 1–100 μmol / L and 0.2–0.6 mmol / L effectively scavenged O_2^- in alkaline DMSO and xanthine / xanthine oxidase systems respectively. The results show that Ber is an antioxidant.

KEY WORDS berbamine; electron spin resonance; luminescence; free radicals; lipid peroxides; antioxidants

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提要 用比色法测定丙二醛 (MDA) 研究小檗胺 (Ber) 抗脂质过氧化作用, 并用化学发光法和自旋捕集技术检测该药对 O_2^- 的清除作用. Ber 11–100 μmol / L 对