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# PROTECTIVE ACTION OF SEVEN NATURAL PHENOLIC COMPOUNDS AGAINST PEROXIDATIVE DAMAGE TO BIOMEMBRANES

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**Abstract**—The effects of seven phenolic compounds isolated from *Salvia miltiorrhiza* on peroxidative damage to liver microsomes, hepatocytes and erythrocytes of rats were studied. The results show that the seven compounds inhibited lipid peroxidation of rat liver microsomes induced by iron/cysteine and Vitamin C/NADPH. The hemolysis of rat erythrocytes induced by hydrogen peroxide was also inhibited. The degree of inhibition varied with different compounds. Among the seven compounds, the action of salvianolic acid A (Sai A) was the most potent. Therefore, the protective action of Sai A against peroxidative damage to isolated rat hepatocytes and their plasma membranes was evaluated further. Malondialdehyde (MDA) production and bleb of the surfaces of rat hepatocytes induced by iron/cysteine were prevented by Sai A. The production of MDA and the consumption of NADPH of the plasma membrane during lipid peroxidation initiated by iron/cysteine and Vitamin C/NADPH were also inhibited. The results strongly suggest that several phenolic compounds like Sai A have a protective action against peroxidative damage to biomembranes.

*Salvia miltiorrhiza* is an important ingredient of certain recipes which have been used widely in traditional Chinese medicine for the treatment of various kinds of diseases. The injection solution and tablets made from *S. miltiorrhiza* are also used by modern medical doctors in China [1] to treat diseases such as cardio-cerebral ischemia and thrombosis. It has been reported that several lipid-soluble substances isolated from *S. miltiorrhiza* and the water-soluble fraction of *S. miltiorrhiza* have multiple pharmacological activities, for instance anti-platelet aggregation and thrombus formation, improving cardio-cerebral circulation [1, 2]. A recent study indicated that the aqueous extract of *S. miltiorrhiza* has scavenging oxygen free radical activity [3]. However, the active components responsible for the antioxidant activity were unknown. Seven phenolic compounds have been isolated from the water-soluble extract of *S. miltiorrhiza* by Li *et al.* [4]. The structures of the seven compounds tested in the present paper are shown in Fig. 1.

This paper reports on the protective action of these seven phenolic compounds against peroxidative damage to liver microsomes, hepatocytes and erythrocytes of rats.

## MATERIALS AND METHODS

*Animals.* Male Wistar rats weighing 150–200 g were used.

*Reagents.* The seven phenolic compounds are salvianolic acid A (Sai A§), salvianolic acid B (Sai B), protocatechualdehyde (Pca), protocatechuic acid (Pro A), caffeic acid (Caa), Danshensu (Das) and rosmarinic acid (Ros). These compounds were supplied by Professor N. L. Li of our Institute. The structures of these compounds were identified with MS, NMR, and i.r. spectra. The purity of the compounds was over 99%. NADPH, cysteine, glutathione and thiobarbituric acid (TBA) were purchased from the Sigma Chemical Co. The other reagents were obtained from the Beijing commercial market.

*Preparation of liver microsomes.* Rats were killed by decapitation. The livers were perfused *in situ* with ice-cold 1% KCl solution from the portal vein. The liver was homogenized in TMS buffer (0.1 M Tris–HCl, pH 7.4, 0.01 M $MgCl_2$, 0.25 M sucrose) [5] with a polytron. The homogenate were centrifuged at 10,000 *g* for 20 min. The supernatant was further centrifuged at 105,000 *g* for 60 min. The pellets were resuspended in TMS buffer and stored at −30° until used.

*Preparation of rat erythrocytes and ghost membrane.* The erythrocytes were obtained from rats by a conventional method. The prepared erythrocytes were resuspended in normal saline at a proportion of 1:1. The ghost membrane was prepared

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§ Abbreviations: Sai A, salvianolic acid A; Sai B, salvianolic acid B; Pca, protocatechualdehyde; Pro A, protocatechuic acid; Caa, caffeic acid; Das, Danshensu; Ros, rosmarinic acid; TBA, thiobarbituric acid; MDA, malondialdehyde; SEM, scanning electron micrograph; SPMS, sucrose–plasma membrane suspension; and TCA, trichloroacetic acid.

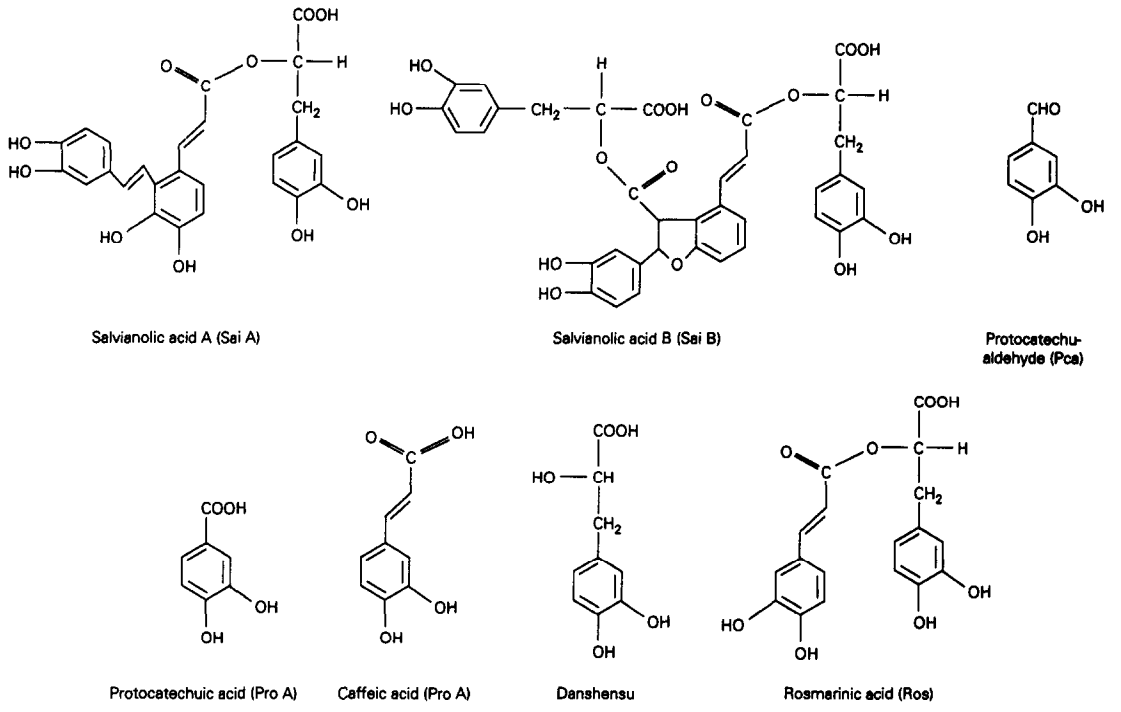


Fig. 1. Chemical structures of seven phenolic compounds isolated from *S. miltiorrhiza.*

Table 1. Effects of seven phenolic compounds on microsomal lipid peroxidation induced by $Fe^{2+}$/cysteine and Vitamin C/NADPH *in vitro*

| | Inhibition of MDA formation (%) | | | | | |
|---|---|---|---|---|---|---|
| | $Fe^{2+}$/cysteine | | | Vitamin C/NADPH | | |
| | Concentration of tested compound (μg) | | | Concentration of tested compound (μg) | | |
| Compound | 25 | 12.5 | 6.25 | 10 | 5 | 2.5 |
| Salvianolic acid A (Sai A) | 97 | 90 | 40 | 93 | 87 | 73 |
| Salvianolic acid B (Sai B) | 87 | 81 | 37 | 73 | 49 | 27 |
| Protocatechualdehyde (Pca) | 77 | 76 | 30 | 87 | 63 | 31 |
| Protocatechuic acid (Pro A) | 35 | 17 | 15 | 15 | | |
| Danshensu (Das) | 42 | 55 | 0 | 23 | | |
| Caffeic acid (Caa) | 45 | 24 | 0 | 27 | | |
| Rosmarinic acid (Ros) | 90 | 74 | 31 | 44 | | |

Each value is the mean of duplicate determinations. The experiment was repeated, and similar results were obtained.

from the washed rat erythrocytes using the method of Jarrett and Penniston [6]. The washed erythrocytes were lysed by the addition of 10 vol. of 10 mM Tris–HCl buffer containing EDTA.

*Preparation of rat hepatocytes.* Rat hepatocytes were isolated with collagenase perfusion according to the method of Seglen [7]. In brief, a small plastic tube was inserted into the portal vein of the rat under ether anesthesia. The liver was removed and circularly perfused with Hanks' buffer containing

Table 2. Effects of four natural phenolic compounds on $H_2O_2$ and MDA-induced hemolysis of rat erythrocytes *in vitro*

| | Inhibition of hemolysis (%) | | | | |
|---|---|---|---|---|---|
| | $H_2O_2$ (2 mM) | | | | MDA (10 mM) |
| | Concentration of tested compound (μg) | | | | Concentration of tested compound (μg) |
| Compound | 20 | 10 | 5 | 2.5 | 20 |
| Sai A | 93 | 95 | 79 | 65 | 24 |
| Sai B | 91 | 67 | 61 | 65 | 0 |
| Pca | 94 | 68 | | | 0 |
| Pro A | 82 | 78 | | | 0 |
| Das | 30 | | | | |
| Caa | 70 | | | | |
| Ros | 65 | | | | |

Each value is the mean of duplicate determinations. The experiment was repeated, and similar results were obtained.

Table 3. Effects of four natural phenolic compounds on depletion of the sulfhydryl group (-SH) induced by $H_2O_2$ (100 mM) in rat ghost membranes *in vitro*

| | -SH group | |
|---|---|---|
| Treatment | nmol/mg protein | % of Control |
| Normal control | 7.8 | 100 |
| $H_2O_2$ | 1.8 | 23 |
| $H_2O_2$ + Sai A | 4.0 | 52 |
| $H_2O_2$ + Sai B | 3.8 | 48 |
| $H_2O_2$ + Pca | 1.8 | 23 |
| $H_2O_2$ + Pro A | 6.6 | 85 |

Each value is the mean of dupliate determinations. The experiment was rejected, and similar results were obtained.

Table 4. Effects of seven phenolic compounds on $Fe^{2+}$/cysteine- and $CCl_4$-induced MDA formation in isolated rat hepatocytes

| Compound (10 μg) | MDA (nmol/$10^6$ cells) | |
|---|---|---|
| | $Fe^{2+}$/cysteine | $CCl_4$ |
| Control | 1.63 ± 0.05 | 2.71 ± 0.32 |
| Sai A | 0.42 ± 0.02* | 1.32 ± 0.08* |
| Sai B | 0.98 ± 0.06† | 2.78 ± 0.46 |
| Pca | 1.23 ± 0.07 | 2.25 ± 0.57 |
| Pro A | 0.56 ± 0.03† | 2.56 ± 0.25 |
| Das | 0.97 ± 0.06† | 2.32 ± 0.31 |
| Caa | 0.89 ± 0.05† | 2.69 ± 0.32 |
| Ros | 0.78 ± 0.06† | 2.31 ± 0.41 |

Each value is the mean of ± SD four determinations.
* $P < 0.01$ vs control.
† $P < 0.05$ vs control.

0.05% collagenase for 15–20 min after perfusion with $Ca^{2+}$- and $Mg^{2+}$-free Hanks' solution for 5 min. The hepatocytes were isolated and collected after filtration, centrifugation and resuspension. The harvest of cells was about $1 \times 10^7$ to $5 \times 10^8$ cells/liver and the viability of hepatocytes was over 90% as determined by the trypan blue dye staining method. The hepatocytes were diluted to $10^6$ cells/mL of RPMI-1640 medium after the incubation; the whole medium and cells were used to measure malondialdehyde (MDA).

*Preparation of hepatocytes for scanning electron micrograph (SEM).* The preparation of a rat hepatocyte specimen for SEM was carried out as follows. A small piece of cover glass was placed on the bottom of a culture containing the hepatocytes. After incubation, the cover glass was removed and underwent fixation, dehydration, critical desiccation and gold spraying. The changes on the surfaces of the hepatocytes were calculated by counting 200 cells at random under a scanning electron microscope.

*Preparation of plasma membrane of rat hepatocytes.* The plasma membrane of livers was prepared from male rats by sucrose gradient centrifugation [8]. Liver homogenate (1%) was prepared with $NaHCO_3$ buffer. Crude plasma membrane suspension was obtained by two successive centrifugations and diluted with 70% sucrose solution to make a 50% sucrose–plasma membrane suspension (SPMS). SPMS was added to the bottom of the centrifuge tubes and layered on top of an equal volume of 45, 41 and 37% sucrose solution successively. The plasma membrane was harvested at the interface between the 41% and 3% sucrose solution after centrifugation at 105,000 *g* for 90 min.

*Measurement of MDA, protein and sulfhydryl (-SH) group.* MDA was determined by the TBA test according to the method of Yagi [9]. Protein determination was performed by the method of Lowry *et al.* [10] The -SH group in the ghost membrane protein was measured by the method of Haest *et al.* [11].

## RESULTS

*Effects of seven phenolic compounds on liver microsomal lipid peroxidation induced by iron/cysteine and Vitamin C/NADPH.* Liver microsomal protein (1.4 mg) in 1 mL of 0.1 M phosphate buffer

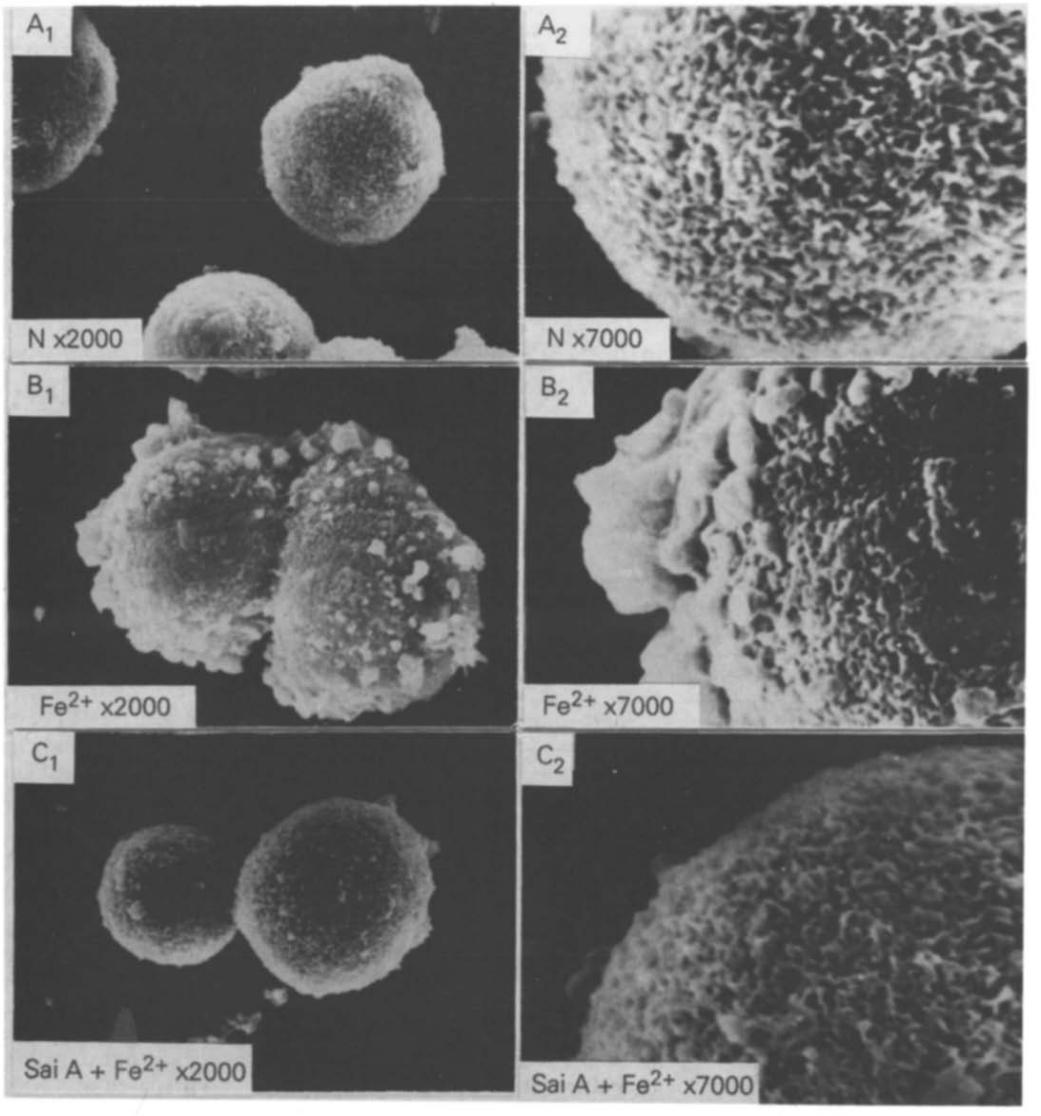


Fig. 2. Scanning electron micrographs of the surfaces of rat hepatocytes: Key: (A) normal hepatocyte, (B) iron/cysteine-treated hepatocyte, and (C) Sai A protected hepatocyte. Left: ×2000. Right: ×7000.

Table 5. Effect of salvianolic acid A (Sai A) on $Fe^{2+}$/cysteine- and Vitamin C/NADPH-induced MDA formation in liver plasma membrane from rats *in vitro*

| Compound (20 μg) | MDA (nmol/mg protein) | |
|---|---|---|
| | $Fe^{2+}$/cysteine | Vitamin C/NADPH |
| Control | 3.06 ± 0.17 | 2.98 ± 0.20 |
| Sai A | 0.89 ± 0.05* | 1.04 ± 0.06* |

Each value is the mean ± SD of four determinations.
* $P < 0.01$ vs control.

(pH 7.4) was incubated with various concentrations of the tested compounds or the same volume of saline for 15 min at 37°. Then lipid peroxidation was initiated by the addition of $FeSO_4$ (50 μM) and cysteine (200 μM) or Vitamin C (250 μM) and NADPH (130 μM). The incubation was carried out for another 30 min. The reaction was stopped by adding 0.5 mL of ice-cold 20% trichloroacetic acid (TCA). Following centrifugation at 3000 rpm for 5 min, the supernatant was used for MDA determination. The percent inhibition of MDA formation was used to express the effect of the tested compound.

The results are shown in Table 1. The seven natural phenolic compounds inhibited MDA production induced by both iron/cysteine and Vitamin C/NADPH at different degrees. Among these compounds, Sai A was the strongest inhibitor of MDA formation. The inhibition by Sai A of MDA production in the Vitamin C/NADPH system was more pronounced than in the iron/cysteine system.

*Effect on hydrogen peroxide induced hemolysis of rat erythrocytes and -SH depletion in ghost*

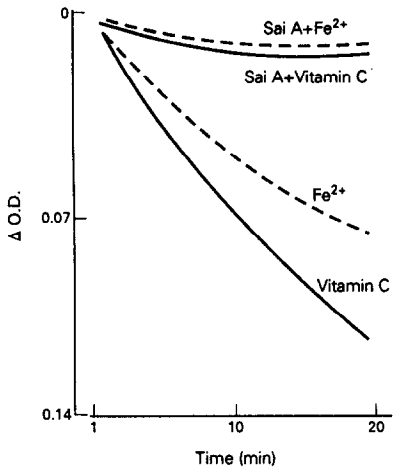


Fig. 3. Effect of Sai A on NADPH consumption by liver plasma membranes during lipid peroxidation induced by iron/cysteine and Vitamin C.

*membranes.* Three millilitres of normal saline containing 0.1 mL of erythrocyte suspension and various concentrations of the tested compounds was incubated in a water bath for 15 min at 37°. After adding $H_2O_2$ (final concentration 2 mM) or MDA (10 mM) to the test tubes, the mixture was reincubated for 30 min. Following centrifugation, the degree of hemolysis was measured by recording the optical density of the suspension at 415 nm on a spectrophotometer.

As shown in Table 2, hemolysis of the erythrocytes induced by $H_2O_2$ was inhibited markedly by the seven compounds at a 20 μg concentration. At a concentration of 10 μg, only four of the seven compounds exhibited an inhibitory effect on hemolysis. Among the four effect compounds, Sai A was shown to be the strongest inhibitor of hemolysis of rat erythrocytes induced by $H_2O_2$. A 20 μg concentration of Sai A showed a weak inhibitory effect on the hemolysis of rat erythrocytes induced by MDA. The other three compounds tested were ineffective.

To study the effects of these compounds on $H_2O_2$-induced depletion of -SH in ghost membranes, 1 mg of the ghost membrane in 1 mL of 0.1 M phosphate buffer (pH 7.4) was incubated with the indicated compound (10 μg) and $H_2O_2$ (100 mM) for 15 min at 37°. Upon completing the incubation, the reaction was ended by adding 10% sodium dodecyl sulfate (SDS). After centrifugation, the -SH group in the supernatant was determined. The four compounds with the stronger activity in inhibition of hemolysis of rat erythrocytes were tested in this experiment. All four compounds at a concentration of 20 μg prevented the depletion of the -SH group induced by $H_2O_2$ in the ghost membrane, although the degree of inhibition varied with the different compounds tested, Sai A was the most potent (Table 3).

*Effect on oxidative damage to the surfaces of rat hepatocytes.* The freshly isolated rat hepatocytes, $1 \times 10^6$ cells/mL of RMPI-1640 medium containing 10% calf serum, were preincubated at 37° for 60 min before adding the tested compounds (each at 25 μg/mL) After incubation for another 60 min, the incubation mixture was changed with fresh medium. The 50 μM $Fe^{2+}$ and 200 μM cysteine were added to the incubation mixture. After incubation for 1 hr, total MDA content in the medium and hepatocytes was determined by the TBA test.

As shown in Table 4, the seven phenolic compounds except for Pca significantly inhibited MDA production. The order of inhibitory potency of the seven compounds was Sai A > Pro A > Ros > caffeic acid > Danshensu > Sai B > Pca. The inhibitory rate varied in the range of 32–79% with the different compounds.

In another experiment of $CCl_4$-induced lipid peroxidation of rat hepatocytes, the incubation procedure of the rat hepatocytes was the same as mentioned above except that 10 μL of $CCl_4$ was used instead of iron/cysteine. The results show that only Sai A inhibited MDA production induced by $CCl_4$, in rat hepatocytes; the other six compounds were ineffective. Therefore, Sai A was selected for further study of protection against damage of the surfaces of rat hepatocytes induced by iron/cysteine. The incubation procedure of the rat hepatocytes was the same as described in Materials and Methods. The changes on the surfaces of the hepatocytes are shown in Fig. 2A–C. The surfaces of normal hepatocytes were smooth and the microvilli were dense and regular (Fig. 2A). When the hepatocytes were damaged by iron/cysteine, the surfaces of almost every cell showed different degrees of changes in shape including large protrusions (Fig. 2B). Pretreatment with Sai A markedly protected the surfaces of the hepatocytes from iron/cysteine-induced injuries; the surfaces appeared smooth with dense and regular microvilli (Fig. 2C).

*Effect on lipid peroxidation of the plasma membrane of rat hepatocytes.* One milliliter of phosphate buffer containing plasma membrane suspension (1 mg protein) was used. The reaction mixture was preincubated with Sai A (20 μg) in a water bath at 37° for 15 min. The reaction was stopped with 20% TCA, and MDA formation in the supernatant of the incubation mixture after centrifugation was determined. The results are shown in Table 5. Sai significantly inhibited MDA production induced by either iron/cysteine or Vitamin C/NADPH in the plasma membrane.

*Effect on NADPH consumption by plasma membranes of rat hepatocytes during lipid peroxidation.* Phosphate buffer (1 mL) containing plasma membrane protein (1 mg) was incubated with $FeSO_4$ (50 μM) and cysteine (200 μM) or Vitamin C (250 μM) in the presence or absence of Sai A (10 μg) in a cuvette at 37°. After the addition of NADPH (380 μM) to the incubation mixture, the change of the absorption value of NADPH at 340 nm was monitored immediately on a UV-spectrophotometer for 20 min. The consumption of NADPH is expressed as the decrease of optical density of the incubation mixture over 20 min.

The changes of the absorption value of NADPH are illustrated in Fig. 3. Owing to lipid peroxidation of the plasma membrane of rat hepatocytes induced

by iron/cysteine and Vitamin C, the consumption of NADPH increased continuously over 20 min as expressed by a decrease of the absorption value at 340 nm. Addition of 10 μg of Sai A almost completely inhibited NADPH consumption by the plasma membrane during lipid peroxidation.

## DISCUSSION

Biomembranes, such as microsomes, erythrocytes and the plasma membrane, are rich in polyunsaturated fatty acids which are very sensitive to peroxidative damage induced by oxygen free radicals. In the present study, iron/cysteine and Vitamin C/NADPH, two generating systems of oxygen free radicals, successfully induced lipid peroxidation of the biomembranes mentioned above. Addition of seven phenolic compounds isolated from *S. miltiorrhiza* inhibited MDA production in liver microsomes and peroxidative damage to the surfaces of rat hepatocytes. The attack of hydrogen peroxide on rat erythrocytes was also counteracted by the compounds. It is very interesting to note that the action of Sai A was more potent than that of the other tested compounds, although they are all phenolic compounds. A question arises as to why Sai A is more active than the other compounds in the protection against peroxidative damage to biomembranes. A possible explanation is that in addition to the configuration of the chemical structure of Sai A, the multiple phenolic hydroxyl groups on its structure may be a determining factor. This speculation is based on the fact that the antioxidant activity of some other compounds, such as Vitamin E and butylated hydroxy toluene, is closely related to the existence of a phenolic hydroxyl group(s) on its structure.

In the experiment on hemolysis of rat erythrocytes, four phenolic compounds with stronger inhibitory effects on MDA formation in liver microsomes and on hemolysis induced by $H_2O_2$ showed no or only a weak effect on hemolysis induced by MDA. In addition, the consumption of NADPH by liver plasma membranes during lipid peroxidation induced by iron/cysteine was inhibited almost completely. These results indicate that the site of protective action of certain compounds isolated from *S. miltiorrhiza* against peroxidative damage may be at the initiation stage of lipid peroxidation of polyunsaturated fatty acid but not at the stage of interaction of the end-product of lipid peroxidation, MDA, with the target cells.

It has been reported that the decoction and injection solution made from *S. miltiorrhiza* exhibited some therapeutic effects on cardiac and cerebral ischemia as well as on liver diseases such as chronic viral hepatitis [1]. It is well known that oxygen free radicals are involved in the damage of reperfusion after organ ischemia [12]. Serum MDA of patients suffering from viral hepatitis is higher than that of normal persons [13]. The present investigation demonstrated that seven phenolic compounds isolated from the aqueous extract of *S. miltiorrhiza* have a strong protective action against oxygen free radical induced peroxidative damage to biomembranes. These phenolic compounds would be the active principles of *S. miltiorrhiza* responsible for antioxidant activity and at least a part of the pharmacological bases for using *S. miltiorrhiza* for the clinical treatment of certain diseases. The authors have also studied the protective action of Sai A against oxygen free radicals and Adriamycin-induced injuries of cardiac mitochondria of rats, as well as the effect of Sai A on the formation of Adriamycin semiquinone radical and oxygen free radicals derived from the reaction of Adriamycin semiquinone with $H_2O_2$ detected by electron spin resonance. The results will be reported in another paper.

## REFERENCES

1. Chen WZ, Pharmacology of *Saliva miltiorrhiza*. *Acta Pharm Sin* **19**: 876–880, 1984.
2. Li CZ, Yang SC and Zhao FD, Effect of tanshinone II-A sulfonate on thrombus formation, platelet and conjunction in rats and mice. *Acta Pharmacol Sin* **5**: 39–42, 1984.
3. Yang WD, Zhu HL and Zhao BL, The scavenging role of oxygen free radicals by *Salvia miltiorrhiza*. *Chin Pharmacol Bull* **6**: 114–117, 1990.
4. Li NL, Tan R and Chen WM, Salvianolic acid A, a new depside from roots of *Salvia miltiorrhiza*. *Planta Med* **50**: 227–228, 1984.
5. Lesca P, Lecointe P, Paoletti C and Mansuy D, Induction des monooxygenase hepatiques par l'ellipticine chez le rat: Formation de cytochrome P-448, activite hydroxylante. *C R Hebd Seances Acad Sci* **282**: 1457–1460, 1976.
6. Jarrett HW and Penniston JT, A new assay for endocytosis in erythrocyte ghosts based on loss of acetylcholinesterase activity. *Biochim Biophys Acta* **448**: 314–324, 1976.
7. Seglen PO, Preparation of isolated rat liver cells. *Methods Cell Biol* **13**: 29–83, 1976.
8. Emmelot P, Isolation of plasma membranes from rats and mouse livers and hepatomas. *Methods Enzymol* **31**: 75–89, 1974.
9. Yagi K, A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem Med* **15**: 212–216, 1976.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Haest CWM, Plasa G, Kamp D and Deuticke B, Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. *Biochim Biophys Acta* **509**: 21–32, 1978.
12. McCord JM, Free radicals and myocardial ischemia: Overview and outlook. *Free Radic Biol Med* **4**: 9–14, 1988.
13. Suematsu T, Liver and serum peroxide levels in patients with liver diseases. In: *Lipid Peroxides in Biology and Medicine* (Ed. Yagi K), pp. 285–293. Academic Press, New York, 1982.