Antioxidative Components of Tanshen (*Salvia miltiorrhiza* Bung)

Ke-Qin Zhang, Yongde Bao, Ping Wu, Robert T. Rosen, and Chi-Tang Ho*

Department of Food Science and The Center for Advanced Food Technology, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903

Rhizomes of tanshen (*Salvia miltiorrhiza* Bung) were pulverized and extracted with diethyl ether. The extract was washed with 5% sodium carbonate solution, freed of solvents, and applied to a silica gel column. Seven colorful bands ranging from light yellow to dark brown were resolved and collected. After certain purification procedures, such as stepwise recrystallization and preparative TLC, seven crystalline quinone-type compounds were obtained, namely tanshinone I, dihydrotanshinone I, tanshinone II_A, tanshinone II_B, methylenetanshinquinone, cryptotanshinone, and danshenxinkun B, all of which had color. Confirmation of the identifications was carried out by mass spectroscopy and infrared spectrometry. Measurement of the antioxidant activity in lard of these quinones was performed with two methods, the Rancimat method and the Shaal oven method in conjunction with peroxide value determination. Except for tanshinone II_A, all the quinones exhibited antioxidant activities, of which dihydrotanshinone I, tanshinone I, methylenetanshinquinone, and cryptotanshinone showed strong activities comparable to that of BHA and BHT. These compounds may have the potential of being used as natural antioxidants in foods and cosmetics.

Tanshen (*Salvia miltiorrhiza* Bung) is an herbal plant. It is normally 1–3 ft tall, covered by a sweep of light yellow fine fuzz. Tanshen has a cylindrical root 8–10 in. long of a brownish red color.

Tanshen is an important herb used frequently in traditional Chinese medicine for its tranquilizing, sedative, circulation-promoting, and bacteriocidal effects (Fang et al., 1976). It is widely used for diseases in gynecology, neurasthenia, hepatomegaly, splenomegaly, and arthritis (Anonymous, 1985). Tanshinone IIA sulfonic acid, sodium salt, a drug made from a quinone contained in tanshen, is now used to treat coronary heart disease.

Studies on the chemical composition of tanshen have been actively conducted, mostly by researchers in the pharmaceutical field (Nakao and Fukushima, 1934; Takiura, 1941a,b; Wessely and Wang, 1940; Wessely and Bauer, 1942; Fang et al., 1976; Okumura et al., 1961; Takiura and Koizumi, 1962; Kakisawa et al., 1968, 1969; Chien et al., 1978; Hayashi et al., 1970a,b, 1971; Baillie and Thomson, 1968; Tateishi et al., 1971; Hayashi and Kakisawa, 1970). Interest has been focused on the crystalline pigments contained in the rhizomes of the plants. A total of 15 compounds of this type have been reported, and the structures are shown in Figure 1.

Some of the clinical effects of tanshen could be, to some extent, related to its possible antioxidant activity; it, however, has not been studied. Research for natural antioxidants has been carried out with great effort. One reason for the wide interest in natural antioxidants is that there are some questions concerning the safety of the presently used synthetic antioxidants (Inatani et al., 1983; Shelef and Chin, 1980; Allen and Engblom, 1972; Branen, 1975; Ito et al., 1985). Most of the identified natural antioxidants fall in the catagories of phenolic and flavonoid compounds. Nevertheless, Houlihan et al. (1985) reported a quinone-type compound, miltirone, from rosemary leaves as having a strong antioxidative effect. It was uncertain at the time as to what functional group in the compound was responsible for the activity. Miltirone has also been found in tanshen among a number of quinone-type compounds, thus raising interesting questions as to whether or not the other quinone in tanshen

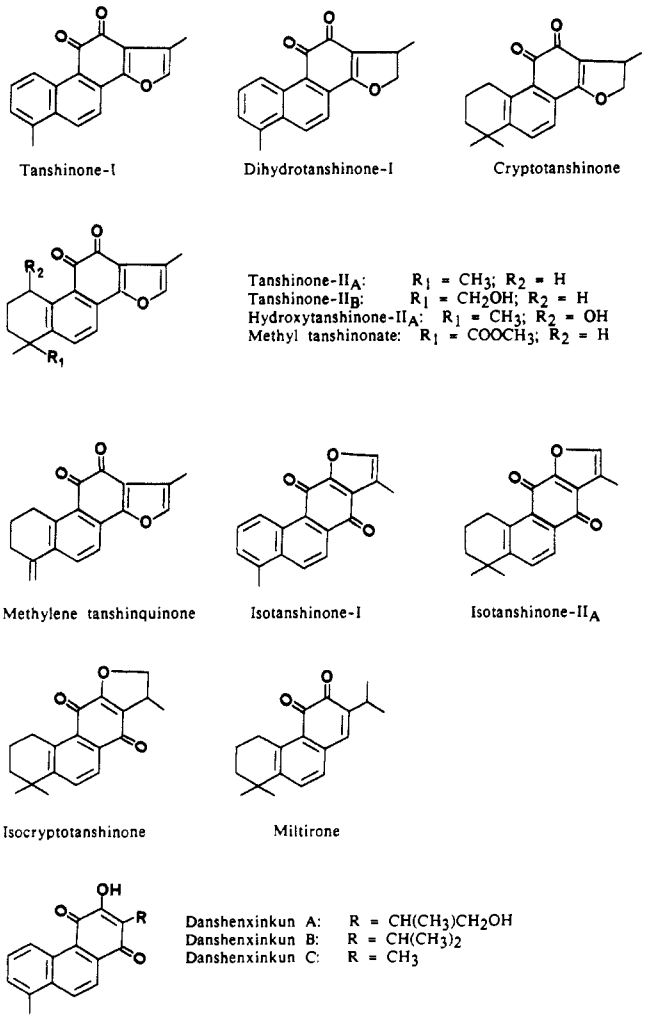

Figure 1. Quinones identified in tanshen.

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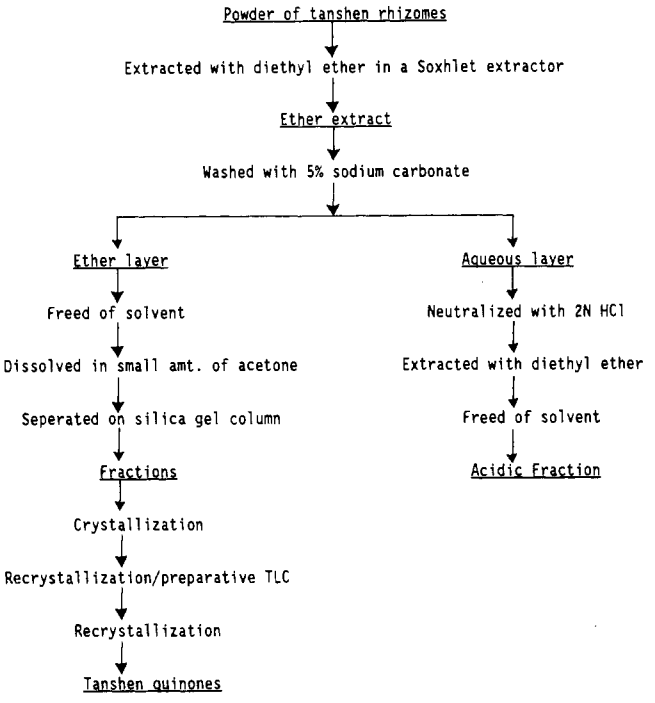
Scheme I. Isolation and Purification of Tanshen Quinones



also has antioxidant activity and how such an activity relates to the structure of the individual compounds.

This paper reports a process of isolate and purify tanshinone I, dihydrotanshinone I, tanshinone II_A, tanshinone II_B, methylenetanshinquinone, cryptotanshinone, and danshenxinkun B from the rhizomes of tanshen and the study of the antioxidant activities of these compounds.

MATERIALS AND METHODS

Materials and Reagents. Tanshen, the rhizomes of the so-called plant, was purchased from a Chinese herbal drug store in Fuzhou City, Fujian Province, People's Republic of China. It was dehydrated and sliced before being commercially available.

Pure pork fat (or lard) without any additives was obtained from the Hatfield Packing Co., Philadelphia, PA.

Isolation and Purification of the Quinone from Tanshen. The overall procedures for isolating and purifying the quinone from tanshen are shown in Scheme I.

(*a*) *Extraction.* The rhizomes of tanshen were mechanically pulverized. Three aliquots of 168 g of the powders were extracted with diethyl ether in a Soxhlet extractor. The extract was washed with 5% sodium carbonate solution and then freed of solvents on a rotary evaporator to prepare it for silica gel column chromatography. The sodium carbonate washings were extracted with diethyl ether to afford the acidic fraction.

(*b*) *Silica Gel Column Chromatography.* The washed extract from (a) was freed of solvent in a rotary evaporator, dissolved in a small amount of acetone, and subjected to column chromatography. A glass column of a diameter 20 mm and length 1000 mm was packed with silica gel (Kieselgel 60, 70–230 mesh ASTM; EM Science, Cherry Hill, NJ) in hexane slurries. The gel had been activated at 200 °C for 12 h before being packed. The column was developed first with diethyl ether, stepwise increasing portions of acetone in ether, stepwise increasing portions of methanol in acetone, and finally pure methanol. The flow rate of the eluents was maintained at 2.5–3.0 mL/min. The distinct colorful bands were collected separately.

(*c*) *Crystallization and Recrystallization.* The color band eluates from the silica gel column were separately concentrated by air current into a hood to obtain crude crystals, which were then, again separately, recrystallized with methanol as the solvent. Repeated operations were needed in some cases to afford pure crystals. The isolated compounds were all confirmed to be over 98% pure by high-performance liquid chromatography.

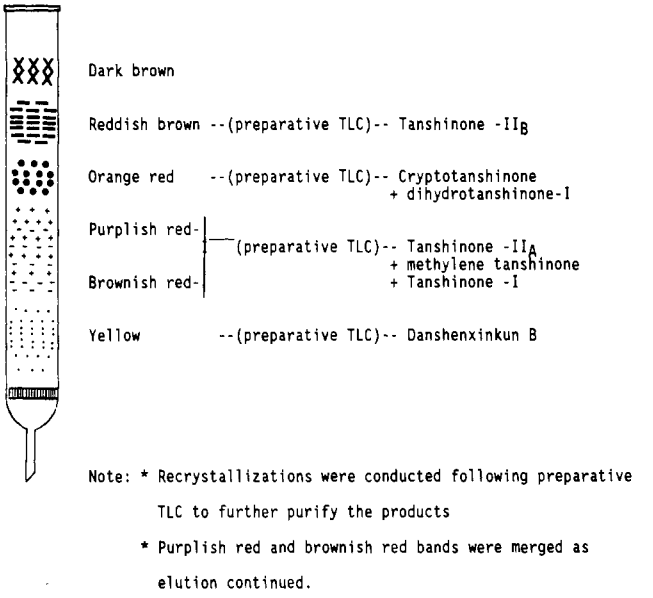


Figure 2. Column chromatographic elution sequence of tanshen quinones.

(*d*) *Preparative Thin-Layer Chromatography.* Silica gel thin-layer plates (Kieselgel $60F_{254}$, 20 × 20 cm, 1-mm thickness, EM Science) were used, onto which the crude products were smeared. The sample plates were then developed with petroleum ether/diethyl ether/acetone (75:20:5). The plates were visualized by the colors of the tanshen components per se or under UV (254-nm) light. The isolated compounds were all confirmed to be over 98% pure by high-performance liquid chromatography.

Structural Confirmation of the Tanshen Components. (*a*) *Infrared Spectroscopy.* Infrared spectroscopy for the tanshen components was performed with a Beckman Acculab-4 infrared spectrometer (Beckman Instruments, Fullerton, CA). Samples were prepared for IR spectroscopy by incorporating the crystals into a potassium bromide pellet. To prepare the pellet, approximately 5 mg of the crystals was mixed with approximately 100 mg of KBr (IR grade; Aldrich Chemical Co., Milwaukee, WI) in an agate mortar and ground to homogeneous fine powders. The pulverized mixtures were then transferred to a dried Wilks Mini-Press to make the pellet.

(*b*) *Mass Spectrometry.* Mass spectrometry for the tanshen components was conducted by electronic ionization (EI) on a VG 7070 EQ mass spectrometer (VG Analytical, Manchester, U.K.), electron energy 70 eV.

Evaluation of the Antioxidant Activity of the Tanshen Components. Pure lard (pork fat, Hatfield Packing Co.) without any additives was used as the substrate to evaluate the antioxidant activity of the components isolated from tanshen. Two methods were employed: the Rancimat method and the Shaal oven method in conjunction with peroxide value determination.

(*a*) *Rancimat Method.* The test samples were prepared by mixing the tanshen components with lard in 0.02% concentration on weight basis. Some commonly used synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbyl palmitate (AP), and propyl gallate (PG) were also used as additives for control.

A 617 Rancimat (METROHM AG, CH-9100 Herisau, Switzerland) was used. A 2.5-g portion of each test sample was loaded into the reaction vessel cylinder. Six different samples were conducted in one batch. The air supply was maintained at 20 mL/min and the heating temperature at 110 °C throughout the experiment.

Determination of the induction time for the test samples was performed by measuring the time span from the beginning to the moment when a sudden change of conductivity occurred.

(*b*) *Schaal Oven Method in Conjunction with Peroxide Value Determination.* The test samples were prepared exactly as stated

Table I. Mass Spectrometric and Infrared Spectroscopic Data of the Tanshen Components

compound	MS: m/z (relative abundance)	IR: wavenumber, cm^{-1}	mp, °C	ref[a]
danshenxinkun B	280 ($M^{\cdot+}$, 100), 265 (9), 247 (10), 237 (12), 219 (8),209 (7), 189 (9), 169 (21)	3257, 2899, 1653, 1631, 1592, 1404, 1351, 1325, 1220, 1130, 865, 790, 778	181–183	1
tanshinone II_A	294 ($M^{\cdot+}$, 100), 279 (52), 261 (87), 251 (73), 233 (19), 222 (14), 178 (20), 165 (33), 152 (16)	2950, 2817, 1686, 1667, 1580, 1534, 1453, 1429, 1403, 1389, 1287, 1193, 1170, 1152, 961, 917, 838, 707	202–204	2, 3, 4
methylenetanshinquinone	278 ($M^{\cdot+}$, 100), 263 (20), 250 (12), 249 (33), 235 (80), 222 (8), 207 (5), 194 (15), 179 (25),178 (34), 165 (25)	2907, 1675, 1667, 1626, 1572, 1534, 996, 959, 840	174–176	5
tanshinone I	276 ($M^{\cdot+}$, 45), 261 (2), 248 (100), 222 (4), 219 (4), 191 (24), 189 (17), 176 (9), 165 (7),152 (4), 139 (3)	1689, 1667, 1595, 1550, 1431, 1333, 1190, 1160, 917, 840, 836, 790, 763, 709	232–233	2, 3, 6
cryptotanshinone	296 ($M^{\cdot+}$, 64), 281 (10), 268 (32), 253 (100), 235 (14), 178 (5), 171 (18), 152 (10)	2857, 1695, 1647, 1626, 1575, 1481, 1441, 1342, 1212, 1176, 1156, 948, 919, 704	174–175	7, 2, 6, 8
dihydrotanshinone I	278 ($M^{\cdot+}$, 17), 250 (55), 235 (100), 222 (4), 207 (10), 189 (8), 179 (26), 169 (9), 165 (9)	2857, 1684, 1653, 1626, 1563, 1481, 1429, 1337, 1304, 1181, 961, 939, 843, 799	223–225	1
tanshinone II_B	310 ($M^{\cdot+}$, 21), 292 (14), 279 (100), 261 (25), 235 (59), 233 (15), 222 (14), 221 (14), 178 (34), 165 (38), 152 (27)	3571, 3472, 2994, 1695, 1667, 1637, 1575, 1531, 1379, 1285, 1160, 917, 841, 670	201–204	3, 8

[a] Key: (1) Fang et al., 1976; (2) Kakisawa et al., 1969; (3) Baillie and Thomson, 1968; (4) Tateishi et al., 1971; (5) Chien et al., 1978; (6) Hayashi et al., 1970; (7) Okumura et al., 1961; (8) Hayashi and Kakisawa, 1970.

Table II. Antioxidant Activity and Yield of the Quinone Isolated from Tanshen

compound	yield, %	induction time, h	peroxide values on day 0	7	14	21	28
weakly acidic fraction	1.12	8.92	1.71	3.56	9.58	50.40	170.71
neutral fraction	0.94	13.58	2.08	4.88	10.07	18.63	28.79
dihydrotanshinone I	0.0086	20.08	1.40	3.34	4.18	5.42	7.64
tanshinone I	0.0384	17.86	2.24	4.32	6.00	8.61	16.10
methylenetanshinquinone	0.0072	14.58	0.66	1.94	3.22	5.62	12.28
cryptotanshinone	0.0548	12.58	1.92	6.06	14.85	29.79	84.87
tanshinone II_B	0.0028	10.90	2.00	3.78	11.13	44.70	132.95
tanshinone II_A	0.0856	4.85	2.08	32.08	131.78	285.20	367.87
danshenxinkun B	0.0012	8.75					
BHT		10.97	0.60	1.70	2.46	3.40	5.37
BHA		18.05	0.60	6.48	13.40	23.20	35.03
ascorbyl palmitate		9.83	0.58	13.11	106.04	225.75	312.15
control (no additive)		4.92	0.60	36.50	128.31	236.92	303.52

for the Rancimat method. A 50-g portion of each sample was placed in a glass jar (the specific area at the beginning was 0.39 cm^2/g) and heated in a gravity convection oven set at 60 ± 1 °C. The peroxides in the samples were measured in duplicates, following the American Oil Chemists' Society's Official Method Cd 8-23, in periods of 0, 7, 14, 21, and 28 days.

RESULTS AND DISCUSSION

Preparation of Tanshen Quinone. When 500 g of tanshen rhizome powders was extracted with ether, 41.5 g of products was obtained, which in turn was extracted with 5% sodium carbonate to afford 5.6 g of the neutral fraction and 4.7 g of the weakly acidic fraction. The water layer remaining was finally extracted with butanol to get 6.2 g of extract and 15 g of water-soluble fraction.

As the neutral fraction of the tanshen extracts was again fractionated on a silica gel column, a spectrum of at least seven colorful bands was observed, ranging from light yellow to red to dark brown. The eluting bands were collected separately and were further purified by either preparative TLC or recrystallization or both in most cases. Figure 2 summarizes the processes and the corresponding products obtained.

Structural Confirmation of the Tanshen Quinone. Structural confirmation of the tanshen components was accomplished by comparing the melting point, mass spectrometric, and infrared spectroscopic data obtained to those published. Table I lists the data found and the references. The melting points and the IR and mass spectra of all the identified compounds matched well with the published data.

Antioxidant Activity of the Tanshen Quinone. Two different methods were employed in investigating the antioxidant activities of the components isolated from tanshen rhizomes, that is, the Rancimat method and Schaal oven method in conjunction with peroxide value determination. When the conductivity changes caused by formation of small molecules of free fatty acids are measured, the Rancimat method is both simpler and easier compared to the active oxygen method (AOM). The correlation between the two methods, which both indicate the induction times of the fats and oils under elevated temperatures and accelerated aeration, has been investigated. Experimental results (Laubli and Bruttel, 1986) showed that the two methods correlated well over a range of temperature (100–120 °C) on a variety of fats and oils, such as peanut oil, sunflower oil, oliver oil, lard, margarine, and cooking butter. However, it should be pointed out that the results were obtained from dealing with the fats or oils alone without antioxidants added. It remains unclear whether any additives would disproportionally favor or suppress the formation of the short-chain fatty acids, therefore rendering the results deviated.

(*a*) *Induction Times.* The induction times (IT) of the tanshen quinone are shown in column 3 of Table II. The longer ITs suggest stronger antioxidant activities. Except for tanshinone II_A, all the tanshen quinones exhibited appreciable activity, led by dihydrotanshinone I, which showed an activity exceeding those of BHA, BHT, or AP in the control group. Other quinones showing activity comparable to BHA and BHT include tanshinone I, methylenetanshinquinone, cryptotanshinone, and tanshinone II_B, in that order.

(*b*) *Peroxide Values.* The peroxide values (PVs) of the lard incorporated with tanshen components incubated

at 65 °C on days 7, 14, 21, and 28 are shown in Table II, as well as the initial PVs of the individual samples for references. At any given moment, stronger antioxidant activities are indicated by lower peroxide values.

Although the peroxide values at any given moment can be used to evaluate the antioxidative power of the additives, the one on day 28, in our opinion, is most indicative. In this case, dihydrotanshinone again showed the highest activity among the tanshen components. In fact, the order of activity established by the Rancimat method was almost repeated by the Schaal oven–peroxide value method except for tanshinone I and methylenetanshinquinone, whose positions in the order interchanged. It was not the only discrepancy found in the experiments. Note that for the controls the Rancimat method suggests BHA being a much stronger antioxidant than BHT while the Schaal oven–peroxide value method does the opposite.

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Registry No. Dihydrotanshinone I, 568-73-0; tanshinone I, 87205-99-0; methylenetanshinquinone, 568-72-9; cryptotanshinone, 17397-93-2; tanshinone II_B, 67656-29-5; tanshinone II_A, 35825-57-1; danshenxinkun B, 65907-76-8.