Extraction and Identification of an Antioxidative Component from Jue Ming Zi (*Cassia tora* L.)

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The antioxidant activity of two cultivars of "jue ming zi" (*Cassia tora* L. and *Cassia occidentalis* L.) was investigated. The results indicated that methanolic extracts from *C. tora* L. (MECT) and *C. occidentalis* L. (MECO) produced stronger antioxidant activity and gave higher yields of extract than other organic solvents. The MECT showed stronger antioxidant activity than did the MECO on peroxidation of linoleic acid. MECT at 200 ppm was stronger than 200 ppm of α-tocopherol, but weaker than 200 ppm of butylated hydroxyanisole. Amberlite XAD-2 column chromatography separated MECT into eight fractions. Of the eight fractions, fraction V possessed significant antioxidant activity and showed 85.8% inhibition on peroxidation of linoleic acid. Subsequently, fraction V was separated into two subfractions, Va and Vb, by Toyoperal HW-40 F gel filtration chromatography. The subfraction Vb exhibited stronger antioxidant activity than did subfraction Va and was identified as 1,3,8-trihydroxy-6-methyl-9,10-anthracenedione (emodin) on the basis of UV−vis spectral, HPLC, IR, MS, and NMR analysis.

Keywords: *Jue ming zi; Cassia tora L.; antioxidant activity; emodin*

INTRODUCTION

Lipid peroxidation is an important deteriorative reaction of foods during processing and storage. Toxic substances formed by lipid peroxidation may lead to other adverse effects such as carcinogensis, mutagenesis, and aging (Yagi, 1987). To avoid or delay this peroxidation process, addition of antioxidants to foods is the most extensive method. Synthetic antioxidants, e.g. butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *tert*-butylhydroquinone (TBHQ), have been used as antioxidants in foods for years. However, consumers are concerned about the safety of synthetic antioxidants (Branen, 1975; Ito et al., 1983). As for tocopherol and ascorbic acid, both are extensively used as natural antioxidants; however, the antioxidant activities of tocopherol and ascorbic acid are lower than those of synthetic antioxidants (Nishina et al., 1991). This concern has resulted in increased interest in the investigation of the effectiveness of naturally occurring compounds with antioxidant properties (Wu et al., 1982; Kikuzaki and Nakatani, 1989; Duh et al., 1992; Yen et al., 1996). The fact that various antioxidants occur naturally in plants has been recognized. They can be observed in fruit, vegetables, nuts, seeds, leaves, flours, roots, and barks (Pratt and Hudson, 1990).

Lipid peroxidation causes destabilization and disintegration of the cell membrane, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer (Rice-Evans and Burdon, 1993). Antioxidants may play a role in preventing the development of vascular disease and some forms of cancer (Halliwell, 1997). The seeds of *Cassia tora* L. and *Cassia occidentalis* L., called jue ming zi in Chinese, have been conventionally used in Chinese medicine for several centuries. Traditionally, jue ming zi has been used to improve visual acuity and to remove "heat" from the liver. Modern physicians use this herb to treat hypercholesterolemia and hypertension. This herb has been reported to contain many active substances, including chrysophenol, emodin, rhein, etc. (Huang, 1993). The roasted jue ming zi has a special flavor and color, and it is popularly used as a health drink tea. Choi et al. (1997) reported that jue ming zi exhibited antimutagenic activity. Moreover, the close relationship between antioxidant activity and antimutagenicity has been demonstrated by Yen and Chen (1995). However, whether these seeds possess other pharmacological effects, e.g. antioxidant activity, remains unclear. Thus, this work investigated the antioxidant activity of jue ming zi and identified its major antioxidative components.

MATERIALS AND METHODS

Material. The seeds of both *C. tora* L. (CT) and *C. occidentalis* L. (CO) were obtained from a local market at Taichung, Taiwan. The seeds of CT and CO were ground into a fine powder in a mill (RT-08, Rong Tsong, Taichung, Taiwan). The ground materials were sealed in a plastic bottle and stored at 4 °C until employed.

Chemicals. Ammonium thiocyanate was purchased from E. Merck (Darmstadt, Germany). Ferrous chloride, BHA, emodin, linoleic acid, α-tocopherol, and Amberlite XAD-2 resin were obtained from Sigma Chemical Co. (St. Louis, MO). Toyoperal HW-40F resin was obtained from Tosoh Chemical Co. (Tokyo, Japan).

Extraction of Antioxidant Components from CT and CO. One kilogram of CT or CO was extracted for 24 h with 1700 mL each of *n*-hexane, ethyl acetate, and methanol,

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respectively, followed by filtration and evaporation of the filtrate to dryness in vacuo, and weighed to determine the yields of soluble constituents and antioxidant activity.

Chemical Analyses. The percentages of moisture, crude protein, crude fat, crude fiber, and ash in jue ming zi were determined according to AOAC (1984) Methods 14.062, 14.067, 14.060, 14.064, and 14.063, respectively.

Determination of Antioxidant Activity. The antioxidant activity of all organic solvent extracts and separated fractions was determined according to the thiocyanate method (Mitsuda et al., 1966). Each sample (1.0 mg) was added to a solution mixture of linoleic acid (2.5 mL, 0.02 M) and potassium phosphate buffer (2 mL, 0.2 M, pH 7.0). The mixed solution, in a conical flask, was incubated at 37 °C. At regular intervals, the peroxide value was determined by reading the absorbance at 500 nm, after reaction with $FeCl_2$ and thiocyanate. The solutions without added organic solvent extracts were used as blank samples. The tests were run in duplicate, and analyses of all samples were run in triplicate and averaged.

Estimation of Anti-$FeCl_2$−H_2O_2-Induced Linoleic Acid Peroxidation. The effect of anti-$FeCl_2$−H_2O_2-induced linoleic acid peroxidation was determined according to the method of Tamura and Shibamoto (1991). A mixture (5 mL) containing methanolic extracts of CT (MECT) or methanolic extracts CO (MECO) (0−1000 ppm, relative to linoleic acid), linoleic acid (0.1 M), $FeCl_2$ (0.4 mM), H_2O_2 (0.4 mM), and phosphate butter (0.2 M, pH 7.4) was incubated at 37 °C for 24 h. After incubation, 1.0 mL of BHT (20 mg/mL), 1.0 mL of thiobarbituric acid (TBA) (1%), and 1.0 mL of HCl (10%) were added to the mixture, which was heated for 30 min on a boiling water bath. After cooling, chloroform (5.0 mL) was added and the mixture centrifuged at 1000*g* to give a supernatant. The absorbance of supernatant was measured spectrophotometrically at 532 nm. A low absorbance value indicated a high antioxidant activity.

Estimation of Anti-$FeCl_2$-Induced Linoleic Acid Peroxidation. The effect of anti-$FeCl_2$-induced linoleic acid peroxidation was determined according to the method of Tamura and Shibamoto (1991). The experimental procedure was the same as above except that the H_2O_2 (0.4 mM) was omitted.

Isolation of the Antioxidants from CT. Column chromatography performed a primary fractionation of the MECT, and gel filtration chromatography was used to isolate and collect the purified compounds.

(a) Column Chromatography. The MECT was fractionated by Amberlite XAD-2 column (6.0 cm diameter and 90.0 cm height; pore diameter = 0.9 μm) chromatography. The extract (70 g) was introduced to the top of the column. The elution was performed by each solvent in the following sequence: water/methanol 100:0; water/methanol 80:20; water/methanol 60:40; water/methanol 40:60; water/methanol 20:80; water/methanol 0:100; methanol/acetone 50:50; and methanol/acetone 0:100. Fractions were collected and their absorbance was measured at 280 nm with a spectrophotometer (Hitachi U-2000, Japan). Eluates were pooled into eight major fractions, solvent was removed under reduced pressure, and the yield and antioxidant activity of each fraction were determined.

(b) Gel Filtration Chromatography. The material of the fraction exhibiting the strongest antioxidant activity among eight fractions was loaded on a 1 m long column (1.6 cm diameter) containing 200 g of resin of Toyoperal HW-40F (Tosoh Co., Japan). The same solvent for the fractionation was employed for elution. Fractions (15 mL) were collected by using an FRAC-100 fraction collector (Pharmacia, Uppsala, Sweden), and their absorbance was measured at 280 nm with a spectrophotometer (Hitachi U-2000, Japan). The collected fractions were pooled into two fractions, solvent was removed under reduced pressure, and the yields and antioxidant activity of each fraction were determined.

UV−Vis Spectrometry. UV−vis absorption spectra of the active components in methanol were recorded on a spectrophotometer (Hitachi U-2000, Japan).

Table 1. Proximate Analysis of *C. tora* L. and *C. occidentalis* L.

composition[a]	*C. tora* L.	*C. occidentalis* L.
moisture (%)	5.83	5.77
crude protein (%)	17.70	17.75
crude fat (%)	5.31	5.24
ash (%)	4.83	4.96
crude fiber (%)	23.97	25.04
N-free extracts[b] (%)	42.36	41.24

[a] Values are means of duplicate analyses. [b] Calculated by differences.

Table 2. Yields and Antioxidant Activities of Extracts from *C. tora* L. (CT) and *C. occidentalis* L. (CO) with Various Solvents

	yield[a] (g; mean ± SD)[b]		antioxidant activity[c] (%; mean ± SD)[b]	
solvent	CT	CO	CT	CO
methanol	0.38 ± 0.02a	0.36 ± 0.03b	76.1 ± 0.003a	71.2 ± 0.008a
ethyl acetate	0.05 ± 0.00c	0.07 ± 0.01c	39.1 ± 0.012c	50.0 ± 0.011b
n-hexane	0.24 ± 0.01b	0.23 ± 0.01b	73.9 ± 0.010b	70.7 ± 0.013a

[a] Based on 5 g of *C. tora* L. and *C. occidentalis* L. [b] Means within a column with the same letters are not significantly different ($P > 0.05$). [c] The antioxidant activity was determined according to the thiocyanate method, and the percent inhibition of linoleic acid peroxidation, 100 − [(Abs increase of sample/Abs increase of control) × 100], was calculated to express antioxidant activity.

High-Performance Liquid Chromatography (HPLC). HPLC analysis was performed with a Hitachi liquid chromatograph (Hitachi Ltd., Tokyo, Japan), consisting of a model L-6200 pump, a Rheodyne model 7125 syringe-loading sample injector, a model D-2000 integrator, and a model L-4200 UV−vis detector set at 276 nm. A Zorbax ODS RP-18 reversed phase column (5.0 μm, 4.6 × 250 mm i.d.; DuPont, Wilmington, DE) was used for analysis. The volume injected was 10 μL. The elution solvents were methanol/water/acetic acid (79:20:1, v/v/v). The flow rate was set at 0.7 mg/mL.

Mass Spectrometry. Mass spectra of active components were recorded by using electron ionization (EI) mode at 70 eV with a JEOL JMS-SX/SX 102A mass spectrometer (Japan). The temperature was raised in steps of 119.7 °C/min from 100 to 300 °C.

IR Spectrometry. The samples were analyzed in KBr pellet, and IR spectral data were obtained by using an infrared spectrophotometer (Hitachi 270-30, Japan).

Nuclear Magnetic Resonance (NMR) Spectrometry. NMR spectra were recorded with a Varian VXR-300S FT-NMR spectrometer (Harbor City, CA) operating at 299.95 MHz for ^{1}H NMR and at 75.43 MHz for ^{13}C NMR with complete proton decoupling. The spectra were observed in CD_3COCD_3. The sweep widths, pulse angles, and repetition rates for ^{1}H NMR were 5500.0 Hz, 7.0 μs, and 0 s, respectively, and for ^{13}C NMR were 22000.0 Hz, 7.0 μs, and 2.0 s, respectively. The chemical shifts are reported in parts per million values from tetramethylsilane.

Statistical Analysis. Statistical analyses were performed according to the SAS (1985) *User's Guide.* Analyses of variance were performed by ANOVA procedure. Significant differences between the means were determined by Duncan's multiple range test.

RESULTS AND DISCUSSION

Table 1 lists the proximate compositions of *C. tora* L. (CT) and *C. occidentalis* L. (CO). Both CT and CO had similar proximate compositions. Among the compositions, the nitrogen-free extract was the highest amount followed by the crude fiber. Table 2 lists the yields and antioxidant activity of extracts from CT and CO with various solvents. Of three solvent extracts, methanolic extracts of CT (MECT) and CO (MECO) displayed the

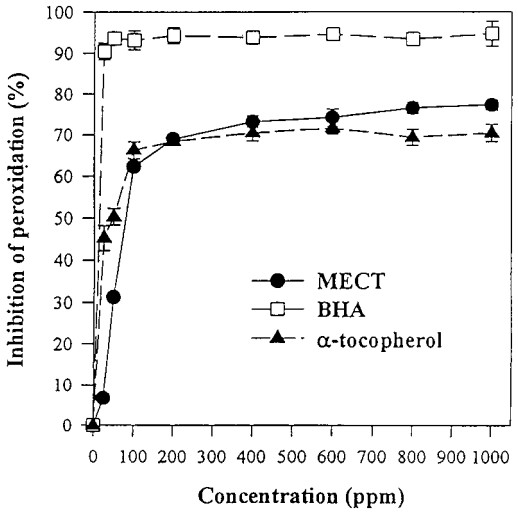

Figure 1. Antioxidant activity of MECT, BHA, and α-tocopherol in the linoleic acid peroxidation system induced by $FeCl_2$ and H_2O_2. The activity was determined by the TBA method.

strongest antioxidant activities, indicating that methanolic extracts produced stronger antioxidant activities and gave higher yields of extract than the other solvents. This observation is in agreement with some reports (Economou et al., 1991; Duh et al., 1992; Tian and White, 1994; Yen et al., 1996) that methanol is a widely used and effective solvent for extraction of antioxidants. MECT and MECO at 200 ppm were less active than 200 ppm of BHA but were stronger than 200 ppm of α-tocopherol (data not shown). MECT at 200 ppm had significantly ($P < 0.05$) stronger antioxidant activity than 200 ppm of MECO (Table 2). The existence of marked antioxidant activity and higher yield was shown in MECT; therefore, MECT was focused on in the following study.

Figure 1 shows the antioxidant activity of MECT in the linoleic acid peroxidation system, compared with BHA and α-tocopherol, induced by $FeCl_2$ and H_2O_2. The antioxidant activity of MECT increased with increasing concentration. At the range of 200−600 ppm, no significant ($P > 0.05$) differences were found between MECT and α-tocopherol, but a significant ($P < 0.05$) difference was observed between MECT and BHA. Iron salts are thought to react with H_2O_2, called the Fenton reaction, to make hydroxyl radicals, which are the most active free radical formed in biological systems (Hochstein and Atallah, 1988) and known to be able to abstract hydrogen atoms from membrane lipids and bring about peroxidic reactions of lipids (Fong et al., 1973; Kidata et al., 1979). From the above results, MECT significantly ($P < 0.05$) inhibits the lipid peroxidation derived from the Fenton reaction, indicating that MECT displayed antioxidant activity in the thiocyanate method as well as the TBA method.

Figure 2 shows the antioxidant activity of MECT in the linoleic acid peroxidation system, compared with BHA and α-tocopherol, induced by $FeCl_2$. The antioxidant activity of MECT increased with increasing concentration up to 200 ppm, and then no significant ($P > 0.05$) differences were shown in antioxidant activity with concentration from 200 to 1000 ppm. In general, the inhibitory effect of MECT in the linoleic acid peroxidation induced by $FeCl_2$ was stronger than that of α-tocopherol when the concentration was >200 ppm. At the range of 0−1000 ppm, the antioxidant activity of MECT

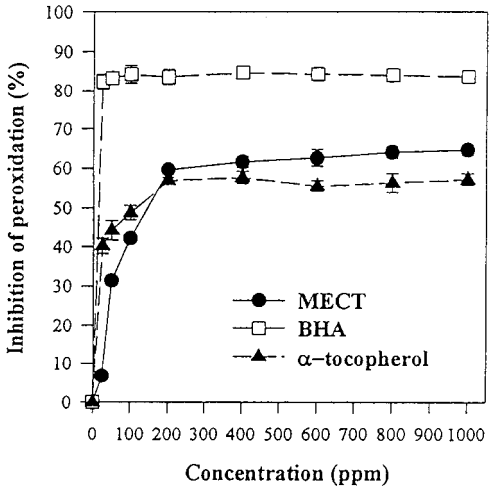

Figure 2. Antioxidant activity of MECT, BHA, and α-tocopherol in the linoleic acid peroxidation system induced by $FeCl_2$. The activity was determined by the TBA method.

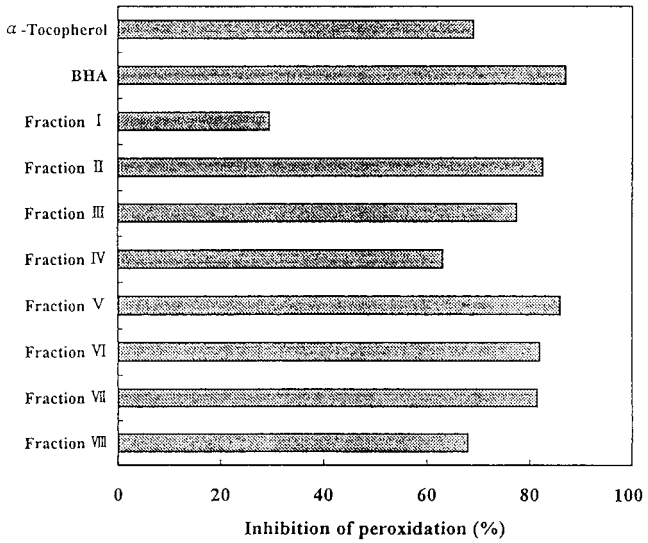

Figure 3. Antioxidant activity of Amberlite XAD-2 column chromatographic fractions obtained from MECT. The activity was determined by the thiocyanate method, and the concentration of each sample was 200 ppm.

was significantly ($P < 0.05$) weaker than that of BHA. Metal ions such as iron or copper may act as prooxidants by electron transfer with liberating radicals from fatty acids or hydroperoxides that promote the lipid peroxidation (Gordon, 1990). The results obtained indicate that MECT significantly ($P < 0.05$) inhibited the linoleic acid peroxidation as compared to the control. In other words, the percentage of inhibition of MECT toward linoleic acid peroxidation was based on the malondialdehyde (MDA) different from the control.

With Amberlite XAD-2 column chromatographic separation, MECT was separated into eight fractions. Figure 3 illustrates antioxidant activity of 200 ppm each of the separated fractions, determined according to the thiocyanate method. Of eight fractions, fraction V displayed the strongest antioxidant activity, with activity equal to that of BHA and much greater than that of α-tocopherol. Fraction V was further purified on Toyopearl HW-40F gel filtration chromatography by using a methanol/water (80:20, v/v) eluent solvent. Two subfractions were observed and designated Va and Vb. Subfraction Vb exerts a more effective antioxidant

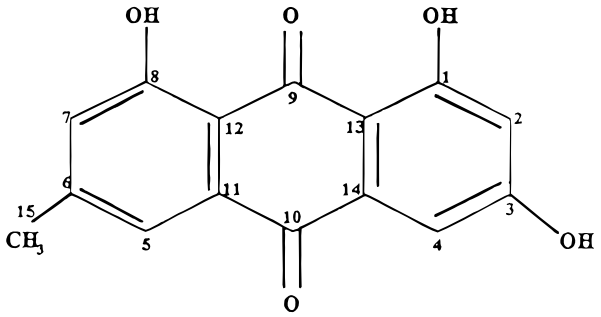

Figure 4. Structure of emodin (1,3,8-trihydroxy-6-methyl-9,10-anthracenedione).

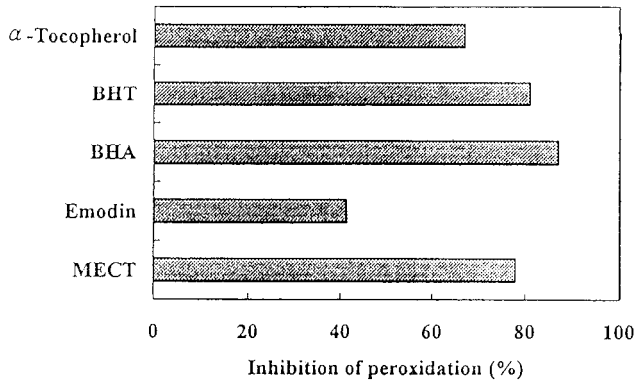

Figure 5. Antioxidant activity of MECT, emodin, BHA, BHT, and α-tocopherol. The activity was determined by the thiocyanate method, and the concentration of each sample was 200 ppm.

activity than subfraction Va (data not shown); therefore, the present investigation of purification and identification was focused more on subfraction Vb.

The spectral λ_{max} value for subfraction Vb in methanol was 276 nm. Therefore, subfraction Vb was determined by HPLC with UV−vis detector set at 276 nm. A distinct peak appeared, and the retention time was 13 min. The IR spectrum showed hydroxyl (3418 cm^{-1}) and carbonyl groups (1632 cm^{-1}). The data of mass spectra (*m/z*) were 270 $(M)^+$, 241 $(M - COH)^+$, and 213 $(M - CO - COH)^+$. The ^1H NMR spectrum of subfraction Vb exhibited 10 hydrogens, with the following chemical shifts: δ 2.466 (3H, s, C_6-CH_3), 6.626 (1H, s, C_7-H), 7.099 (1H, s, C_2-H), 7.207 (1H, s, C_5-H), 7.616 (1H, s, C_4-H), 12.029 (2H, s, C_1-OH and C_3-OH), 12.152 (1H, s, C_8-OH). The ^{13}C NMR spectrum revealed 15 peaks, with the following chemical shifts: δ 166.397 (C_1), 114.450 (C_2), 163.344 (C_3), 109.643 (C_4), 124.998 (C_5), 149.627 (C_6), 124.900 (C_7), 166.317 (C_8), 191.810 (C_9), 182.214 (C_{10}), 134.228 (C_{11}), 108.878 (C_{12}), 121.526 (C_{13}), 136.631 (C_{14}), 21.940 (CH_3). On the basis of these data, a molecular formula of $C_{15}H_{10}O_5$ was proposed, and this was identified as 1,3,8-trihydroxy-6-methyl-9,10-anthracenedione (emodin). Figure 4 represents the structure of emodin. Moreover, the antioxidant activity of purified subfraction Vb also was compared with that of authentic emodin, which was determined according to the thiocyanate method, and no significant ($P > 0.05$) difference was found between purified subfraction Vb (40.7 ± 1.1%) and authentic emodin (41.5 ± 0.3%).

Figure 5 shows the antioxidant activity of emodin compared with those of MECT and commercial antioxidants α-tocopherol, BHA, and BHT. Emodin was less effective than α-tocopherol, MECT, BHA, and BHT. Emodin was demonstrated as an antioxidative component of MECT. Hence, the antioxidant activity of emodin was less than that of MECT, likely because MECT was fractionated. The marked antioxidant activity of MECT may be contributed to by emodin and other minor antioxidative component in MECT.

Dziedzic et al. (1984) reported that polyphenols generally display not only marked antioxidant activity toward fat oxidation but also the presence of a carbonyl group in the molecule, which appears to be necessary for a high level of antioxidant activity. On the basis of Figure 4, emodin contains three hydroxyl groups and two carbonyl groups. Hence, emodin possesses an antioxidant activity. In addition, emodin contributed to the antioxidant activity of the extract of CT, although several minor components in the extract also showing antioxidant activity were not identified. Further research on the mechanism and application of the antioxidant effects of MECT is in progress.

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