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Squalene Content and Antioxidant Activity of *Terminalia catappa* Leaves and Seeds

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Squalene was identified by gas chromatography−mass spectrometry and high-performance liquid chromatography (HPLC) spiking analyses in the supercritical CO_2 extracts of freeze-dried abscisic leaves of *Terminalia catappa* L. When the freeze-dried abscisic, senescent, mature, and immature leaves and seeds were subjected to supercritical CO_2 extraction at 40 °C and 3000 psi and HPLC quantitation, squalene contents were 12.29, 2.42, 1.75, 0.9, and 0% in the extracts and corresponding to 1499, 451, 210, 65, and 0 μg/g in the freeze-dried sample, respectively. When the extracts were applied for antioxidative characterization by supplementation in an iron/ascorbate system with linoleic acid and in a pork fat storage system for inhibition of conjugated diene hydroperoxide (CDHP) formation or in a free radical scavenging system with 1,1-diphenyl-2-picryl-hydrazyl (DPPH), the extracts of leaves exhibited potent antioxidative and DPPH scavenging activities and increased with an increase of leaf maturity. However, the seed extracts only exhibited potent inhibition of CDHP formation and very low DPPH scavenging activity.

KEYWORDS: ***Terminalia catappa*** **L.; supercritical CO_2 extraction; squalene; antioxidant activity; GC-MS**

INTRODUCTION

Terminalia catappa L. (*Combretaceae*, *Terminalia* Linn.) grows commonly in tropical and subtropical regions, particularly localized in the seashore or coastal areas. Leaves and fruits of *T. catappa* have been used as folk medicines for antipyretic and hemostatic purposes and prevention of hepatoma and hepatitis (*1*−*3*). In general, the water extracts prepared after cooking dried leaves of *T. catappa* for a prolonged period are ready to use as a folk medicine. The water extracts of *T. catappa* leaves can suppress CCl_4-induced hepatotoxicity in male Wistar albino rats and are of potency in treatment of all chemical-induced hepatotoxicity (*2*, *3*). Punicalin and punicalagin have been identified in the water extracts of *T. catappa* leaves as anti-AIDS compounds (*4*, *5*). Tannin and flavonoid glycosides of *T. catappa* leaves to exhibit a significant free radical scavenging effect and inhibition of Cu^{2+}-induced LDL oxidation have been reported (*6*−*8*). Haemagglutinating and trypsin inhibitor activities have been detected in the seed extract of *T. catappa* (*9*). Wang et al. (*10*) used a distillation−solvent system to extract and characterize the antioxidative properties of the essential oils from the leaves of *T. catappa*, and 35 compounds have been identified. Squalene has not been reported in the extracts of *T. catappa*.

Squalene (**Figure 1**) is a triterpenic hydrocarbon, a known natural antioxidant, and has been found in the deep sea shark liver and olive oils (*11*, *12*). After intake, it is usually transported and stored in the skin tissue and plays a role in releasing oxidative stresses, such as sunlight exposure. Squalene may inhibit HMG-CoA reductase, lower farnesyl pyrophosphate availability in prenylation of *ras* oncogene, and interfere with transducing activity of *ras* gene (*12*). The quenching reaction with singlet oxygen by squalene in a model system has also been demonstrated (*13*). Shark liver oil is a potent source of squalene and is used as a major ingredient in formulation of a health food named "shinkaizame-ekisu" (deep sea shark liver oil) in Japan (*11*). Squalene from shark liver is a popular folk medicine for chronic diseases of the liver (*14*, *15*). Antitumor and anticarcinoma activities of squalene have also been reported (*16*). The traditional source for squalene is primarily from shark and whale liver oil (*11*, *12*). The use of squalene in cosmetic applications is limited due to uncertainty of its availability as a result of international concern for protection of the marine animals. This consideration turned our interest toward a plant source of squalene.

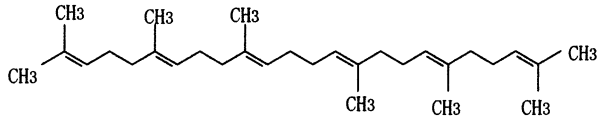

Figure 1. Chemical structure of squalene.

Supercritical fluid extraction (SFE) is effective and powerful in extraction of the compounds of interest from solid materials at a fairly low temperature. Supercritical carbon dioxide fluid is commonly used as a solvent because of its low supercritical temperature, its ease in elimination of residual solvent, and its

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nontoxicity as well as nonflammability. In particular, supercritical CO_2 extraction is efficient in extraction of compounds with low polarity. In this study, the immature, mature, senescent, and abscisic leaves and seeds of *T. catappa* were subjected to supercritical CO_2 extraction. After they were extracted, the predominant compounds in the extracts were identified and the extracts were further subjected to antioxidative characterization.

MATERIALS AND METHODS

Samples of *T. catappa* L. and Supercritical CO_2 Extraction. Immature, mature, senescent, and abscisic leaves and seeds from the ripened fruits were collected from *T. catappa* trees on the campus of National Chiayi University in the fall season (September to November). The trees are over 15 years old. The collected samples were cut into pieces, freeze-dried, ground into fine powders, sealed in polyethylene plastic bags, and stored at −25 °C for use.

An Isco 100 DX supercritical fluid extractor (Isco Technology, Lincoln, NE) using CO_2 as a supercritical fluid solvent was used. Aliquots (1.0 g) of the samples were loaded into a series of extraction vessels (10 mL) and subjected to supercritical CO_2 extractions. A composite design of extraction condition including 40, 50, and 60 °C of temperature and 2000, 3000, and 4000 psi of pressure was conducted. The static and dynamic extraction times were, respectively, run for 15 min, and the flow rate was 3 mL/min. After they were extracted, the extracts were weighed to determine the extraction yields and stored at −25 °C for further analyses.

Analyses of the Supercritical CO_2 Extracts with Gas Chromatography (GC), GC−Mass Spectrometry (GC−MS), and High-Performance Liquid Chromatography (HPLC). The supercritical CO_2 extracts and 1-hexanol (internal standard) were dissolved in 5 mL of pentane and subjected to GC analysis. The analysis followed the method of Wang et al. (*10*) with minor modifications. An HP 6890 gas chromatograph coupled with a flame ionization detector (Hewlett-Packard Co., Palo Alto, CA) with a 30 m × 0.25 mm i.d., 0.25 μm, HP-Inno-Wax capillary column was used. Helium was used as the carrier gas, and the flow rate was 1 mL/min. The oven temperature was programmed from 40 to 230 °C at 2 °C/min and held at 230 °C for 30 min. Both temperatures of injection port and detector were 250 °C. The injection volume and split ratio were 1 μL and 15:1, respectively.

For presumptive identification, an HP 5973N GC−MS (Hewlett-Packard Co.) was used. The operating condition of the GC for MS analysis was identical to that of GC analysis described above. For MS analysis, the separation temperature and ionization voltage were 265 °C and 70 eV, respectively. Mass spectra and the reconstructed chromatograms were obtained by automatic scanning of the samples in the mass range of *m*/*z* 50−550. Chromatographic peaks were checked for homogeneity with the aid of mass chromatograms with characteristic fragment ions. Compound identification was tentatively verified according to the data system of Wiley 275 mass spectral library (Hewlett-Packard Co.).

An L-7100 HPLC pump in connection with an L-7420 UV/vis detector monitored at 215 nm (Hitachi Co. Ltd., Tokyo, Japan) and a reverse phase column, 250 mm × 4.6 mm i.d., 5 μm, Thermal Hypersil ODS column (Thermo Hypersil GmnH, Kleinostheim, Germany) was operated with an isocratic solvent condition. The mobile phase was *n*-butanol/methanol (35:65, v/v) and degassed by passing through an LDG-2410 solvent degasser (Uniflows Co., Tokyo, Japan). Each supercritical CO_2 extract was dissolved in *n*-butanol at 50 μg/mL and applied for HPLC analysis. The flow rate, injection volume, and sample loop were 1 mL/min, 40 μL, and 20 μL, respectively.

For identification of squalene with HPLC in the supercritical CO_2 extracts, in addition to the presumptive identification with GC and GC−MS analyses described above, the supercritical CO_2 extract solution (50 μg/mL dissolved in *n*-butanol) was spiked with an equal volume of *n*-butanol containing an authentic standard of squalene (Sigma Chem. Co., St. Louis, MO) (50 μg/mL) and subjected to HPLC analysis for comparison.

Antioxidative Potency (AOP) Determinations of the Supercritical CO_2 Extracts. For determination of the AOP with linoleic acid in an iron/ascorbate system, Tris buffer (50 mM, 20 mL) sonicated with 0.1 g of linoleic acid and 1.0 g of Tween-20 was used as substrate. Then, 0.5 mL of the substrate was supplemented with 0.1 mL of a supercritical CO_2 extract solution, butylated hydroxytoluene (BHT), and squalene (25, 50, or 100 μg/mL dissolved in *n*-butanol) and 0.5 mL of a prooxidant solution (30 μM $FeCl_3$ and 200 μM ascorbic acid). The mixtures were incubated at 37 °C for 5 and 30 min. After the mixtures were incubated, the reactant was mixed with 10 μL of 4% BHT in MeOH (w/v) to stop further oxidation. Then, the mixture (0.1 mL) was mixed with 2.4 mL of MeOH and subjected to absorbance determination at 234 nm with an UV spectrophotometer as a measure of conjugated diene hydroperoxide content. Aliquots (0.1 mL) of *n*-butanol without antioxidant or containing 1000 μg/mL BHT were introduced and incubated concurrently as blank and negative controls, respectively. Extent of peroxidation was determined by dividing the absorbance increase between 5 and 30 min of incubation with a supercritical CO_2 extract by that of incubation without antioxidant (blank). Thus, AOP expressed as a percentage was calculated as follows:

$$\text{AOP} = (1 - \Delta 234\text{ nm with antioxidant}/\Delta 234\text{ nm without antioxidant}) \times 100$$

For antioxidative characterization of the supercritical CO_2 extract with pork fat system, a previously reported procedure (*17*) was followed. For pork fat preparation, a series of 60 g ground pork fat patties placed in porcelain bowls were cooked for 2 h in a forced-air oven set at 125 °C. After they were cooked and cooled to ambient temperature, aliquots (1.5 mL) of the top layer oils were withdrawn and deposited into 1.5 mL microfuge tubes and centrifuged (8000*g* for 1 min). Aliquots (1.0 mL) of the oil were deposited in a series of 20 mL brown vials and supplemented with 0.1 mL supercritical CO_2 extract, BHT, and squalene solutions (100 μg/mL in *n*-butanol) and placed in an oven set at 60 ± 1 °C for 6 days. During storage, 2.5 μL of oil was withdrawn periodically and dissolved in 2.5 mL of isooctane and the absorbance at 234 nm was measured spectrophotometrically. The conjugated diene hydroperoxide content was expressed as absorbance units at 234 nm of a 0.1% (v/v) oil solution in isooctane. Oils prepared from the ground pork fat patty cooked alone were stored and determined as blank.

Free Radical Scavenging Activity Determination. For determination of the free radical scavenging activity with 1,1-diphenyl-2-picrylhydrazyl (DPPH), the method reported by Shimada et al. (*18*) was followed. DPPH solution (2 mM in *n*-butanol) was freshly prepared as a free radical source. A series of supercritical CO_2 extract and squalene solutions (100, 200, 300, and 500 μg/mL in *n*-butanol) and 100 and 200 μg/mL of BHT in *n*-butanol were prepared. Each 2.5 mL of the supercritical CO_2 extract or BHT solution was mixed with 0.25 mL of DPPH solution and incubated at room temperature (25 ± 2 °C) for 30 min. After the extracts were incubated, the absorbance of the solution at 517 nm was determined. As a control, 2.5 mL of *n*-butanol was mixed with 0.25 mL of DPPH solution and incubated concurrently. The percentage of DPPH free radical scavenging activity is expressed as

$$\text{scavenging activity} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100$$

Statistics and Replicates. Triplicate experiments were conducted, and means of the determinations with standard deviation are reported. The paired samples *t* test was applied for statistical analyses by SPSS 8.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

When the supercritical CO_2 extracts (extracted at 3000 psi and 40 °C) of *T. catappa* abscissed leaves were subjected to GC and GC−MS analyses, 24 compounds (including 1-hexanol as an internal standard) were separated and presumptively identified (**Table 1** and **Figure 2**). A predominant compound was detected at a retention time of 94.68 min and presumptively identified as squalene. Its weight percentages in proportion to the weight of supercritical CO_2 extracts and the weight of freeze-

Table 1. Presumptive Identification of the Comprising Components in the Supercritical CO_2 Extracts (Extracted at 3000 psi and 40 °C) of the Abscisic Leaves of *T. catappa* by GC–MS Analysis and Library Scanning

peak no.	retention time (min)	identified compound (presumptive)	contents in the extract (μg/g)[a]	contents in the sample (μg/g)[a]
1	17.68	1-hexanol	IS	IS[b]
2	29.09	furfural	2275	27.53
3	37.76	imidazo(1,5-a) quinoxaline	2303	27.86
4	56.72	linalyl acetate	2532	30.64
5	61.24	heneicosane	6191	74.92
6	63.69	2-pentadecanone	4735	57.30
7	65.57	2-heptyl-2-undecenal	8754	105.92
8	69.73	linalool	11 350	137.34
9	73.75	octadecane	13 860	167.71
10	75.95	tetracosane	7297	88.29
11	77.62	pentacosane	13 887	168.03
12	78.61	hexacosane	7267	87.93
13	81.34	1-hexacosene	14 028	169.74
14	84.99	2-hexadecen-1-ol	11 569	139.98
15	88.55	heptacosane	19 010	230.02
16	89.50	ionone	7361	89.07
17	90.36	hexadecanoic acid	3326	40.25
18	91.99	4,8,12,16-tetramethyl-heptacosane	6277	75.96
19	94.68	squalene	96 715	1170.25
20	95.82	2,4-bis(dimethylbenzyl)-6-*tert*-butylphenol	16 632	201.25
21	96.39	octacosane	28 887	349.53
22	100.04	9-octadecenoic acid	7752	93.81
23	101.67	9,12-octadecadienoic acid	6406	77.51
24	108.70	ethyl linoleolate	10 302	124.66

[a] Content was quantitated based on the internal standard, and results are means of three extracted samples. [b] IS, internal standard.

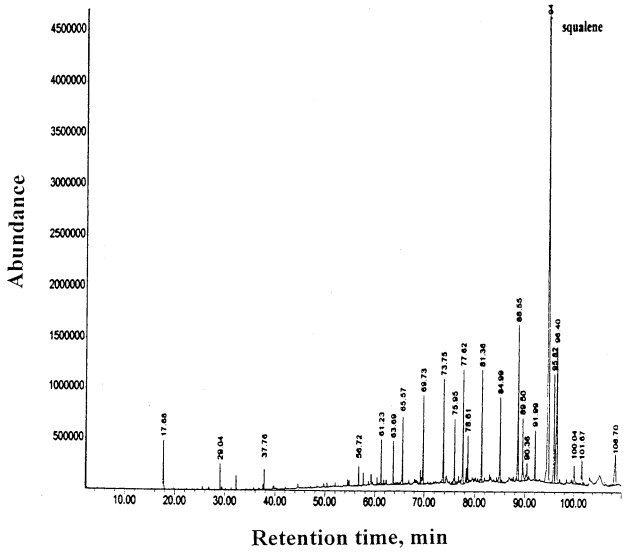

Figure 2. GC of the supercritical CO_2 extracts (extracted at 3000 psi and 40 °C) of the abscisic leaves of *T. catappa*; the separated compounds were identified by GC–MS analysis and library scanning. The peak with 94.68 min of retention time was presumptively identified as squalene.

dried leaf sample were 9.67% (96715 μg/g) and 0.12% (1170 μg/g), respectively. In addition to squalene detected in the supercritical CO_2 extracts, other presumptively identified compounds higher than 100 μg/g included 2-heptyl-2-undecenal, linalool, octadecane, pentacosane, 1-hexacosene, 2-hexadecen-1-ol, heptacosane, 2,4-bis(dimethylbenzyl)-6-*tert*-butylphenol, octacosane, and ethyl linoleolate. Because squalene is a well-known natural antioxidant, its presence in the supercritical CO_2 extracts of *T. catappa* leaves has attracted our interest and attention.

For further identification, the presumptive squalene in the supercritical CO_2 extracts was spiked with an authentic standard of squalene and subjected to HPLC analysis. A significant enlargement of the target peak was observed after spiking (**Figure 3**). This is a first report of the presence of squalene in the leaves of *T. catappa*. Squalene has long been known as one of the major constituents in the shark liver and is also present in olive oils (*11*, *12*). In the report of Wang et al. (*10*), who used distillation solvent by a Likens−Nickerson device to extract the essential oils from *T. catappa* leaves, squalene, probably due to its high molecular weight and low volatility, or its destruction during distillation at an elevated temperature, was not included in the 35 identified compounds. In addition, squalene was not reported as being extracted by water in other investigations (*1*, *3*, *5*−*8*), possibly due to its low polarity.

When the abscissed leaves of *T. catappa* were subjected to supercritical CO_2 extraction under various pressures and temperatures and followed by HPLC analyses, squalene concentrations were higher in the extracts obtained at 2000 psi and 40, 50, and 60 °C or at 3000 psi and 40 °C than in the other extracts (**Figure 4**). At each extraction temperature, squalene contents in the extracts decreased with an increase of pressure. When the different supercritical CO_2 extracts were subjected to GC−MS analysis, the chromatograms and the presumptively identified compounds (data not shown) varied quantitatively rather than qualitatively as affected by extraction condition.

When the freeze-dried immature, mature, senescent, and abscisic leaves and seeds of *T. catappa* were subjected to supercritical CO_2 extraction at 3000 psi and 40 °C, the highest extraction yield was obtained from the seeds, while the highest squalene content was found in the extract of the abscissed leaves (**Table 2**). The squalene contents were 0.90, 1.75, 2.42, 12.29, and 0% (w/w) in the extracts, corresponding to 65, 210, 451, 1499, and 0 μg/g in proportion to the freeze-dried samples, respectively. Apparently, squalene content in the leaves increased with an increase of maturity. Unexpectedly, squalene was not detected in the seeds of *T. catappa* whereas the highest extraction yield as an oily product was obtained from the seeds. The difference of squalene content in the supercritical CO_2 extracts of the abscissed leaves shown in **Tables 1** and **2** may

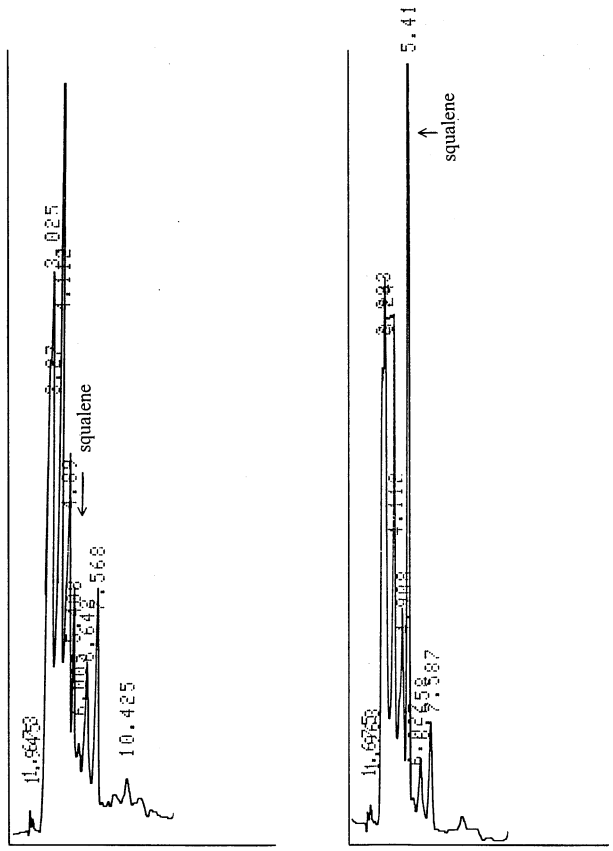

Figure 3. HPLC chromatograms of the supercritical CO_2 extracts before and after spiking with an authentic standard. (A) Supercritical CO_2 extracts (50 μg/mL) and (B) spiked with an equal volume of authentic squalene (50 μg/mL).

be due to the difference of methodology between GC−MS and HPLC.

When the supercritical CO_2 extracts of *T. catappa* were subjected to AOP determination with linoleic acid in an iron/ascorbate system, extracts of the abscissed leaves exhibited the highest AOP among the extracts of leaves and seeds (**Figure 5**). When comparisons were made at the same concentration levels, including 25, 50, and 100 μg/mL, all AOP values of the supercritical CO_2 extracts were higher than those of BHT and squalene. It is of merit to observe the potent AOP of the supercritical CO_2 extracts higher than that of BHT, a proved food supplement of antioxidant. Because the AOP of supercritical CO_2 extracts was higher than pure squalene, other components in addition to squalene in the supercritical CO_2 extracts may also contribute to the overall AOP performance. Referred to the presumptively identified compounds shown in **Table 1**, linalool and 2,4-bis(dimethylbenzyl)-6-*tert*-butylphenol are known antioxidants. Similarly, squalene was not detected in the extracts of seeds, but the extracts exhibited a potent antioxidative activity; the extracts must contain some unidentified natural antioxidants.

When a series of pork fats were supplemented with the supercritical CO_2 extracts of *T. catappa* and subjected to storage at 60 °C for 6 days, CDHP contents increased significantly after 3 days of storage as affected by the supplemented concentration (**Figure 6**). On the basis of the increases from 3 to 6 days of storage, the highest conjugated diene hydroperoxide content was observed in the blank (control oil) and followed in order by the oils supplemented with squalene, the extracts of immature leaves, mature leaves, seeds, senescent leaves, abscisic leaves, and BHT, respectively. Apparently, the supercritical CO_2 extracts exhibited potent activity in inhibition of pork fat peroxidation and the potency varied as affected by plant matrix and leaf maturity.

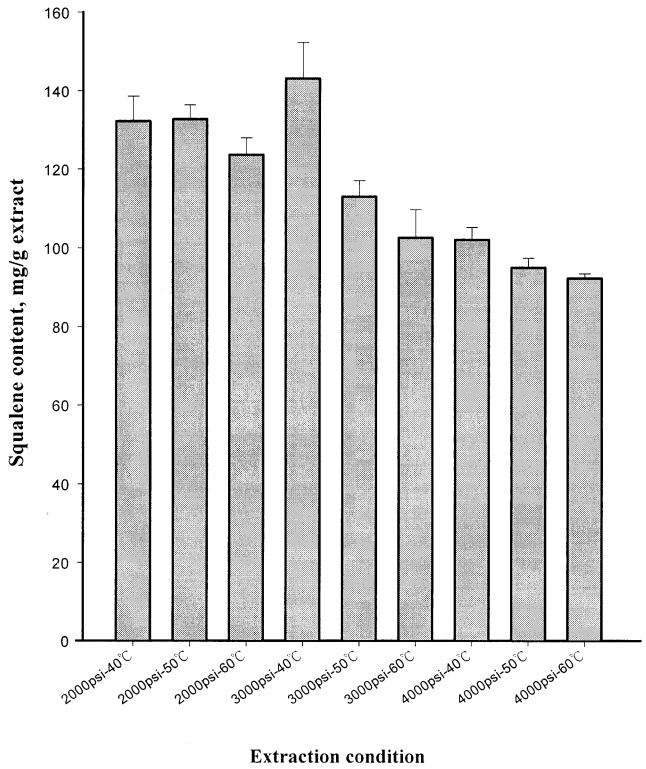

Figure 4. Squalene concentrations in the supercritical CO_2 extracts of the abscisic leaves of *T. catappa* as affected by various extraction conditions. Results are mean ± standard deviation ($n = 3$).

Table 2. Supercritical CO_2 Extraction Yields and Squalene Content (Extracted at 3000 psi and 40 °C) of the Immature, Mature, Senescent, and Abscisic Leaves and Seeds of *T. catappa*

extraction source	extraction yield (mg/g sample)[a]	squalene content in the extract (% (w/w))[a]	squalene content in the sample (μg/g)[a]
immature leaves	7.3[d]	0.90[d]	65[d]
mature leaves	12.0[c]	1.75[c]	210[c]
senescent leaves	18.6[b]	2.42[b]	451[b]
abscisic leaves	12.2[c]	12.29[a]	1499[a]
seeds	179.2[a]	nd	nd

[a] Results are means of determinations ($n = 3$); a value in a column followed by the same letter is not significantly different at the 5% level.

When the supercritical CO_2 extracts of *T. catappa* were introduced at various concentrations for determination of DPPH free radical scavenging activity, with the exception of seeds, the dose−response of the supplemented concentration to increase the free radical scavenging activity was obvious (**Figure 7**). When comparisons were made on the same level of 200 μg/mL, the highest activity was achieved by BHT, followed in order by the extracts of abscisic leaves, senescent leaves, mature leaves, immature leaves, seeds, and the authentic squalene. The DPPH free radical scavenging activity increased with an increase of leaf maturity. Because squalene is a singlet oxygen scavenger (*13*) and unable to scavenge DPPH (**Figure 6**), the observed free radical scavenging activity of *T. catappa* leaves might be caused by other extracted components. On the basis of the

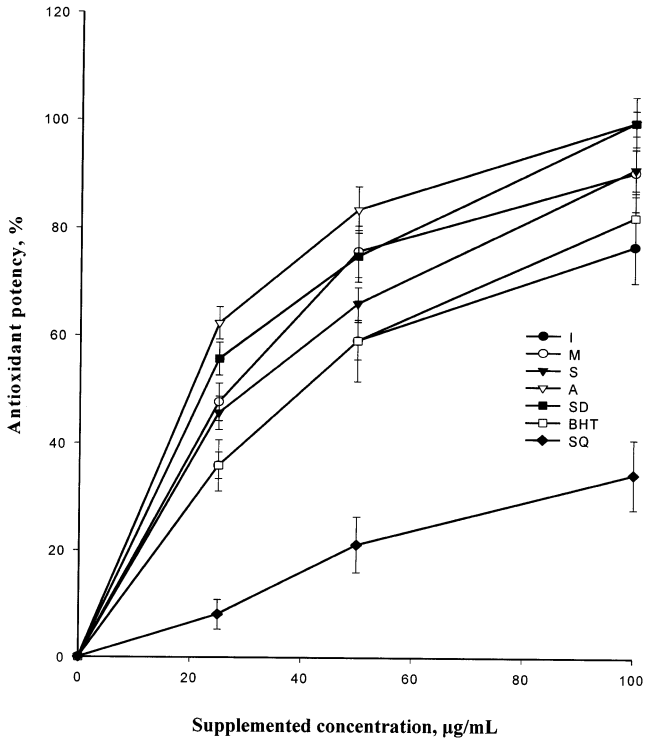

Figure 5. Antioxidative potency of the supercritical CO_2 extracts of *T. catappa* leaves and seeds determined with linoleic acid peroxidized in an iron/ascorbate system. I, immature; M, mature; S, senescent; A, abscisic leaves; SD, seeds; BHT, butylated hydroxytoluene; SQ, squalene. Results are mean ± standard deviation ($n = 3$).

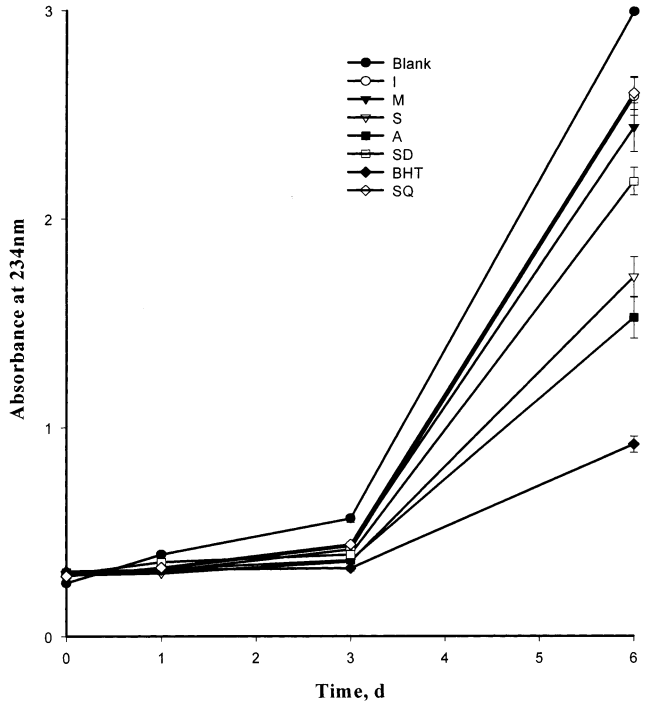

Figure 6. Changes of the conjugated diene hydroperoxide contents during storage of the pork patty oils supplemented with the supercritical CO_2 extracts of *T. catappa* leaves and seeds at 60 °C for 6 days. I, immature; M, mature; S, senescent; A, abscisic leaves; SD, seeds; BHT, butylated hydroxytoluene; SQ, squalene. Results are mean ± standard deviation ($n = 3$).

fact that seed extract did not contain squalene and did not exhibit DPPH scavenging activity (<10%) even supplemented at 500

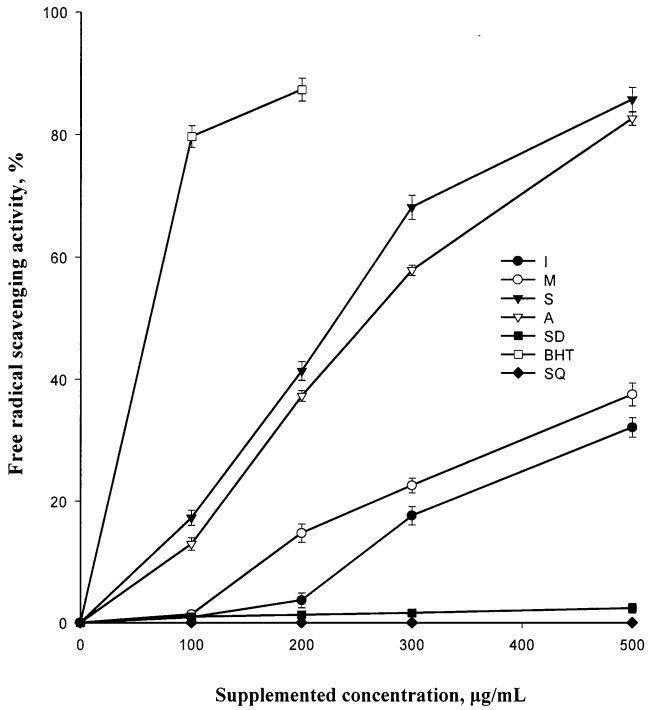

Figure 7. Free radical scavenging activities determined with DPPH of the supercritical CO_2 extracts of *T. catappa* leaves and seeds. I, immature; M, mature; S, senescent; A, abscisic leaves; SD, seeds; BHT, butylated hydroxytoluene; SQ, squalene. Results are mean ± standard deviation ($n = 3$).

μg/mL, the mechanism of the observed antioxidative activity of seed extracts in inhibition of CDHP (**Figures 5** and **6**) must be different from that of leaf extracts.

In conclusion, both supercritical CO_2 extracts (extracted at 3000 psi and 40 °C) from the leaves and seeds of *T. catappa* exhibited a potent antioxidative activity against CDHP formation. Because activity of the extracts was higher than that of pure squalene (**Figures 5** and **6**) and, in fact, squalene was not detected in the seed extracts (**Table 2**), other extracted components also played roles contributing to the observed antioxidative performance. When the freeze-dried leaves with various maturities and seeds of *T. catappa* were subjected to supercritical CO_2 extraction, the extraction yield and squalene content in the extracts obtained from abscisic leaves were 12.2 mg/g and 12.29%, respectively (**Table 2**). Trees of *T. catappa* are widespread and, in particular, produce tremendous amount of absicsic leaves each year. On the basis of the fact that considerable amounts of squalene and other antioxidative components could be extracted by supercritical CO_2, a favorite and efficient method from the viewpoint of environmental protection, these leaves are a potential source of squalene and other medicinal phytochemicals. This study has opened an interesting field of worthy further investigations addressed on the nature and functionality of the extracted components in addition to squalene.

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