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Antioxidant properties of solvent extracts from *Terminalia catappa* leaves

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Abstract

Solvent extracts were prepared from green, yellow fallen and red fallen leaves of *Terminalia catappa* L. and their antioxidant activities were evaluated. Other antioxidant properties of methanolic extracts, including reducing power, scavenging and chelating effects, were also determined. The yields were consistently in the order of yellow fallen (6.34–10.50%) > red fallen (5.12–9.98%) > green leaf extracts (2.36–6.08%) for four solvents used. Higher yields were obtained from extraction with ethyl acetate or methanol than with dichloromethane or pentane. For three different leaves, the antioxidant activities were in the order methanol > ethyl acetate > dichloromethane > pentane extracts and all showed a parabolic-like curve with the maximum at 0.1–0.5 mg ml^{-1} of solvent extract. Reducing powers of three methanolic extracts and their scavenging effects on 1,1-diphenyl-2-picrylhydrazyl radicals were excellent at 0.5 and 0.1 mg ml^{-1}, respectively. At 30 mg ml^{-1}, chelating effects of methanolic extracts from green, yellow fallen and red fallen leaves on ferrous ions were 77.3, 48.6 and 48.3%, respectively.

Keywords: *Terminalia catappa*; Antioxidant activity; Reducing power; Scavenging effect; Chelating effect

1. Introduction

Oxidation in living organisms is essential for the acquirement of energy to proceed biological processes. However, oxygen-centred free radicals and other reactive oxygen species, that are continuously produced in vivo result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell & Gutteridge, 1999). Although almost all organisms possess antioxidant defence and repair systems, that have evolved to protect them against oxidative damage, these systems are insufficient to entirely prevent the damage (Simic, 1988). However, antioxidant supplements, or foods containing antioxidants, may be used to help human body reduce oxidative damage.

Terminalia catappa L. (Combretaceae) is used commonly as a folk medicine in Taiwan, and has been claimed to have therapeutic effects for liver related diseases (Lin & Kan, 1990). Traditionally, only the fallen leaves of *T. catappa* were boiled in water and used as a drink. The leaves of *T. catappa* contain many hydrolysable tannins, such as punicalagin, punicalin, terflavins A and B tergallagin, tercatain, chebulagic acid, geraniin, granatin B, and corilagin, but no caffeine (Tanaka, Nonaka, & Nishioka, 1986). Punicalin and punicalagin showed inhibited HIV replication in infected H9 lymphocytes with little cytotoxicity and also in purified HIV reverse transcriptase (Nonaka et al., 1990). Kashiwada, Nonaka, Nishoka, Chang, and Lee (1992) further indicated that chebulagic acid and geraniin exhibited moderate selective cytotoxicity against RPMI-795 melanoma cells.

Liu et al. (1996) reported that the water extract of the dried leaves of *T. catappa* inhibited lipid peroxidation in vitro and TPA-induced hydrogen peroxide formation in human mononuclear leukocytes, depending on the dosage used. It was reported that the anticlastogenic effect of *T. catappa* leaves might be attributed to their antioxidant potential (Liu et al., 1996). In addition, Chen, Li, Liu, and Lin (2000) found that the water extract from fallen leaves and punicalagin were effective against bleomycin-induced genotoxicity in Chinese hamster ovary cells. Chen et al. (2000) also indicated that the effectiveness

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could be, at least in part, due to their antioxidant potential.

Although many synthetic chemicals, such as phenolic compounds are found to be strong radical scavengers, they usually have side effects (Imaida, Fukushima, Sivai, Ohtani, Nakanishi, & Ito, 1983). Antioxidant substances obtained from natural sources will be of great interest. The high contents of tannins in *T. catappa* leaves reveals that they may serve as a source of natural antioxidants. Therefore, our objective was to evaluate the antioxidant activity of various solvent extracts from green, yellow fallen and red fallen leaves of *T. catappa* using dichloromethane, ethyl acetate, methanol and *n*-pentane. Other antioxidant properties of methanolic extracts from three different leaves, including reducing power, scavenging effect on 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radicals, and chelating effect on ferrous ions, were also determined.

2. Materials and methods

2.1. Plant material

Three different leaf samples (green, yellow fallen and red fallen leaves) were collected at three distinct periods of time: June–July, September–October and November–December in the campus of the Hung-Kuang Institute of Technology, Taichung, Taiwan. The identity of the plant was confirmed by Dr. Sy-Chian Liu (Department of Botany, National Chung-Hsing University, Taichung, Taiwan). These voucher specimens were deposited at the Department of Food and Nutrition, Hung-Kuang Institute of Technology.

Collected *T. catappa* leaves were carefully washed with deionised water to eliminate extraneous matter and immediately dried in an oven at 40 °C for 12 h. After a fine powder (20 mesh) was obtained using a mill (Retsch Ultra Centrifugal Mill and Sieving Machine, Haan, Germany), dichloromethane, ethyl acetate, methanol or *n*-pentane was used as an extractant, respectively. The extraction of each ground sample (20 g) was conducted in a Soxhlet apparatus, continuously for 12 h with 50 ml of *n*-pentane, dichloromethane, ethyl acetate or methanol at 45, 50, 85, and 70 °C, respectively. The extracts were filtered, evaporated to dryness in vacuo and weighed to determine the yield of extracted constituents. The dried extracts from three different leaves were redissolved in the originally used solvent to a concentration of 50 mg ml^{-1} and stored at −20 °C for further use.

2.2. Antioxidant activity

The antioxidant activity was determined according to the 1,3-diethyl-2-thiobarbituric acid (DETBA) method (Furuta, Nishiba, & Suda, 1997; Suda, Furuta, & Nishiba, 1994). To 50 μl of each leaf extract (0.01–5 mg ml^{-1}) in the originally used solvent (*n*-pentane, dichloromethane, ethyl acetate or methanol) were added 50 μl of linoleic acid emulsion (Sigma Chemical Co., St. Louis, MO, 2 mg ml^{-1} in 95% ethanol). The mixture was incubated in an oven at 80 °C for 60 min, and cooled in an ice bath. To the mixture were, sequentially, added, 200 μl of 20 mM butylated hydroxytoluene (BHT, Sigma), 200 μl of 8% sodium dodecyl sulphate (SDS, Merck Co., Darmstadt, Germany), 400 μl of deionised water, and 3.2 ml of 12.5 mM DETBA (Aldrich Chemical Co., Milwaukee, WI) in 0.125 M sodium phosphate buffer (pH 3.0). The mixture was mixed thoroughly, placed in an oven at 95 °C for 15 min, and then cooled with an ice bath. After 4 ml of ethyl acetate were added, the mixture was mixed and centrifuged at 1000×***g*** at 20 °C for 15 min. The ethyl acetate layer was separated and its absorbance measured in a Hitachi 650–40 spectrofluorometer with fluorescence excitation at 515 nm and emission at 555 nm. The antioxidant activity was expressed as per cent inhibition of lipid peroxidation, with a control containing no sample being 0%. A higher percentage indicates a higher antioxidant activity. α-Tocopherol (20 mM) was used as a control.

2.3. Reducing power

The reducing power was determined according to the method of Oyaizu (1986). Each leaf extract (0.01–5 mg ml^{-1}) in methanol (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6, Wako Pure Chemical Co., Osaka, Japan) and 2.5 ml of 1% potassium ferricyanide (Sigma), and the mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v, Wako) was added, the mixture was centrifuged at 200×***g*** for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% ferric chloride (Wako), and the absorbance was read at 700 nm in a Hitachi U-2001 spectrophotometer. A higher absorbance indicates a higher reducing power. Vitamin E (α-tocopherol), vitamin C (ascorbic acid) and butylated hydroxyanisole (BHA) were also used as controls.

2.4. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical

Each leaf extract (0.01–5 mg ml^{-1}) in methanol (4 ml) was mixed with 1 ml of methanolic solution containing a final 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) radical concentration of 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Vitamin E (α-tocopherol), vitamin C (ascorbic acid) and butylated hydroxyanisole (BHA) were also used as controls.

2.5. Scavenging effect on hydroxyl radicals

The hydroxyl radical reacted with the nitrone spin trap 5,5-dimethyl pyrroline-N-oxide (DMPO, Sigma) and the resultant DMPO-OH adduct was detected with an electron paramagnetic resonance (EPR) spectrometer. The EPR spectrum was recorded 2.5 min after mixing 200 μl of each leaf extract (0.01–5 mg ml^{-1}) in methanol with 200 μl of 10 mM H_2O_2 (Merck), 200 μl of 10 mM Fe^{2+} (Sigma) and 200 μl of 10 mM DMPO using a Bruker EMX-10 EPR spectrometer (Rheinstetten, Germany) set at the following conditions: 3480-G magnetic field, 1.0 G modulation amplitude, 0.5 s time constant, and 200 s scan period (Shi, Dalal, & Jain, 1991). Vitamin E (20 mM) was used as a control.

2.6. Chelating effects on ferrous ions

Chelating effect was determined according to the method of Shimada et al. (1992). To 2 ml of the mixture, consisting of 30 mM hexamine (Wako), 30 mM potassium chloride (Sigma) and 9 mM ferrous sulphate (Union Chemical Works, Hsinchu, Taiwan), were added 2 ml of each leaf extract (0.01–5 mg ml^{-1}) in methanol and 200 μl of 1 mM tetramethyl murexide (TMM, Sigma). After 3 min at room temperature, the absorbance of the mixture was determined at 485 nm. A lower absorbance indicates a higher chelating power. Ethylenediaminetetraacetic acid (EDTA) was used as a control.

2.7. Statistical analysis

For solvent extracts from *T. catappa* leaves, three samples were prepared for assays of antioxidant activity. For methanolic extracts from *T. catappa* leaves, three samples were prepared for other antioxidant attributes, including reducing power, scavenging and chelating effects. The experimental data were subjected to analysis of variance for a completely random design as described by Steel, Torrie, and Dickey (1997) to determine the least significant difference at the level of 0.05.

3. Results and discussion

3.1. Antioxidant activity

Following extraction with various solvents, ethyl acetate extracted more oleoresin from yellow fallen and red fallen leaves of *T. catappa* than the other three solvents whereas methanol extracted more oleoresin from green leaves (Table 1). Generally, using four different solvents, the yields were consistently in the order of yellow fallen (6.34–10.50%) > red fallen (5.12–9.98%) > green leaf extracts (2.36–6.08%). Overall, the higher yields were obtained from extraction with ethyl acetate or methanol for three different leaves.

Using the DETBA method, the oleoresins extracted from green leaf, yellow fallen and red fallen leaves of *T. catappa* showed 29.4–95.7% inhibition of peroxidation (Table 2). However, at 20 mM (8.6 mg ml^{-1}), vitamin E inhibited the peroxidation of linoleic acid by 96.6%. In dichloromethane extracts, a higher inhibition of peroxidation (>85%) was observed for yellow fallen and red fallen leaves at 0.5 mg ml^{-1} and for the three different leaves at 0.1 mg ml^{-1}. In ethyl acetate extracts, better antioxidant activities (>85%) were observed for yellow fallen and red fallen leaves at 0.1 and 0.5 mg ml^{-1}. In methanolic extracts, better antioxidant activities (>85%) were observed for the three different leaves at 0.1 and 0.5 mg ml^{-1}. In pentane extracts, a higher inhibition of peroxidation were found for yellow fallen and red fallen leaves at 0.5 mg ml^{-1} and for yellow fallen leaves at 0.1 mg ml^{-1}.

Generally, for the three different leaves, the antioxidant activities by the DETBA method were higher with the oleoresins extracted with more polar solvents and in the order methanol > ethyl acetate > dichloromethane > pentane extracts. Surprisingly, as shown in Table 2, the antioxidant activities of extracts all showed a parabolic-like curve with a maximum at 0.1–0.5 mg ml^{-1} of solvent extract. Evidently, at concentrations below 0.1–0.5 mg ml^{-1}, the antioxidant activity increased as the concentration increased whereas at concentrations above that, the antioxidant activity decreased. It was anticipated that

Table 1
Yields of oleoresins extracted from *Terminalia catappa* leaves with various solvents

Solvent	Yield[a] (%)		
	Green leaves	Yellow fallen leaves	Red fallen leaves
Dichloromethane	c2.36±0.45C	a6.75±0.77C	b5.12±0.36D
Ethyl acetate	b4.25±0.20B	a10.50±0.58A	a9.98±0.44A
Methanol	b6.08±0.33A	a9.08±0.16B	a8.85±0.32B
n-Pentane	b3.97±0.27B	a6.34±0.38C	a5.89±0.19C

[a] Each value is expressed as mean±S.D. ($n=3$). Means with different capital letters within a column are significantly different ($P<0.05$). Means with different small letters within a row are significantly different ($P<0.05$).

Table 2
Antioxidant activity of oleoresins extracted from *Terminalia catappa* leaves

Oleoresin	Leaves[a]	Inhibition of peroxidation[b] (%)			
		5 mg ml^{-1}	0.5 mg ml^{-1}	0.1 mg ml^{-1}	0.01 mg ml^{-1}
Dichloromethane	G	56.2±2.35B	75.3±1.88C	85.2±0.38C	43.5±8.53A
	Y	69.1±1.93A	93.1±0.78A	94.8±0.40A	31.9±3.26A
	R	62.7±1.24AB	85.8±0.53B	90.4±0.76B	31.4±1.72A
Ethyl acetate	G	34.5±2.69B	70.8±0.28C	81.2±2.21B	36.7±2.21C
	Y	59.2±3.72A	91.4±0.44A	92.5±0.76A	56.7±3.92B
	R	42.4±1.25B	88.9±0.43B	92.1±1.77A	75.2±7.80A
Methanol	G	73.6±3.08A	89.6±0.94A	92.2±0.93B	63.4±2.76C
	Y	69.7±1.06A	90.7±1.07A	95.7±0.33A	88.0±1.60A
	R	76.1±2.62A	90.3±1.06A	95.4±0.63A	79.8±1.35B
n-Pentane	G	67.9±3.27A	83.4±0.36B	83.9±5.20A	36.6±1.14AB
	Y	72.0±2.33A	90.7±1.70A	85.7±1.59A	42.0±1.30A
	R	68.7±2.73A	90.4±0.41A	69.6±2.92B	29.4±3.40B

[a] G, green leaves; Y, yellow fallen leaves; R, red fallen leaves.
[b] Each value is expressed as mean±SD ($n=3$). Means with different capital letters within the column of a specific solvent are significantly different ($P<0.05$).

solvent extracts from three different leaves of *T. catappa* acted as antioxidants, at low concentrations. However, at higher concentrations, solvent extracts showed a pro-oxidant effect. Since the methanolic extracts from three different leaves consistently showed the highest antioxidant activities and the overall higher antioxidant activity was observed for methanolic extracts, this revealed that the antioxidant components might be effectively extracted by methanol. Accordingly, only the methanolic extracts were prepared thereafter for evaluation of other antioxidant properties of *T. catappa*.

3.2. Reducing power

Reducing powers of methanolic extracts from three different leaves were excellent at concentrations as low as 0.5 mg ml^{-1} (Fig. 1). At 0.1 mg ml^{-1}, reducing powers of the three methanolic extracts were in the range 0.48–0.66, comparable with that of vitamin E (0.45), but much less than that of BHA and vitamin C (0.98 and 1.04, respectively). However, at 0.5 mg ml^{-1}, reducing powers of the three methanolic extracts, vitamins C and E and BHA were all comparable (1.05–1.15). The reducing powers of methanolic extracts from *T. catappa* leaves might be due to their hydrogen-donating abilities, as described by Shimada et al. (1992). Accordingly, the methanolic extracts from *T. catappa* leaves might contain a higher amount of reductone, which could react with free radicals to stabilise and terminate radical chain reactions.

Ko (1998) studied antioxidant properties of water extracts from three different leaves of *T. catappa* and found that, at 0.5 mg ml^{-1}, reducing powers of water extracts from green and yellow fallen leaves were 1.28 and 1.15, respectively, higher than those of methanolic extracts from the same leaves in Fig. 1 (1.05 and 1.09).

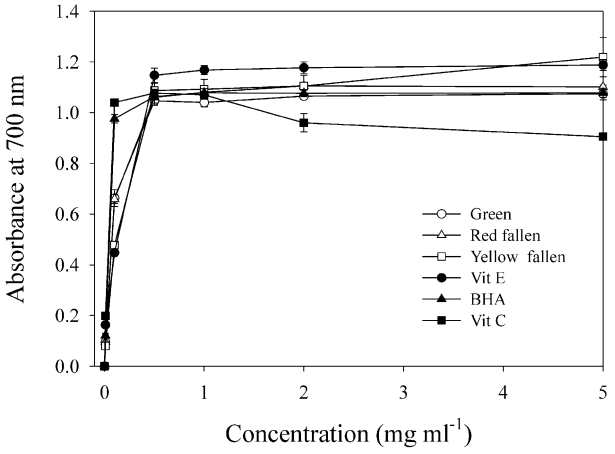

Fig. 1. Reducing power of methanolic extracts from *Terminalia catappa* leaves. Each value is expressed as mean±standard deviation ($n=3$).

However, at 0.5 mg ml^{-1}, the reducing power of water extract from red fallen leaves was 0.89, lower than that of methanolic extract from red fallen leaves in Fig. 1 (1.06). Obviously, the compositions of water and methanolic extracts were different and were not consistent for the three different leaves. To understand the discrepancy in reducing powers by some components in water and methanolic extracts from the three different leaves, the composition of these two extracts require further studies.

3.3. Scavenging effect on 1,1-diphenylhydrazyl and hydroxyl radicals

Scavenging effects of three methanolic extracts on DPPH radicals were excellent at concentrations as low

as 0.1 mg ml^{-1} (Fig. 2). At 0.1 mg ml^{-1}, the scavenging effects of the three methanolic extracts were 92.5–95.7% and comparable to those of vitamins C and E and BHA (95.2–96.7%). Ko (1998) found that at 5 mg ml^{-1}, water extracts from green, yellow fallen and red fallen leaves of *T. catappa* scavenged DPPH radicals by 52.1, 41.4 and 41.1%, respectively. However, at 50 mg ml^{-1}, the scavenging effects of water extracts from green, yellow fallen and red fallen leaves were 76.0, 92.4 and 66.5%, respectively (Ko, 1998). Evidently, the methanolic extracts from the three different leaves were superior over the water extracts in scavenging DPPH radicals.

At 0.2 mg ml^{-1}, scavenging effects on hydroxyl radicals were 58.2, 56.1 and 74.8% for methanolic extracts from green, yellow fallen and red fallen leaves, respectively. However, at 20 mM (8.6 mg ml^{-1}), vitamin E exhibited a scavenging ability of 43.8%. Ko (1998) showed that scavenging abilities on hydroxyl radicals were in the range 72.9–78.2% for three water extracts prepared from 3, 5, 10 or 15 min boiling of the three different leaves or 15 min stirring at room temperature. However, the per cent solids in the water extracts were not provided by Ko (1998). Therefore, a comparison of water and methanolic extracts was not made. In addition, Wang, Ko, Chyau, Mau, and Kao (2000) found that the essential oils from three different leaves of*T. catappa* scavenged hydroxyl radicals by 40.4–51.3% at 12.5 μl and 69.2–82.5% at 50 μl.

Okamoto, Hayase, and Kato (1992) indicated that glycated protein had a scavenging ability for hydroxyl radicals. Shi et al. (1991) have reported scavenging activity of hydroxyl radicals by caffeine, and attributed the reported anticarcinogenic properties of caffeine to this activity. Furthermore, Yen and Hsieh (1995) pointed out that the ability of xylose–lysine Maillard reaction products to quench the hydroxyl radicals was directly related to their antimutagenicity. Therefore, it was anticipated that the three methanolic extracts from *T. catappa* leaves might also possess antimutagenic properties.

3.4. Chelating effect on ferrous ions

Chelating effects of the methanolic extracts from green leaves on ferrous ions increased from 35.7% at 5 mg ml^{-1} to 72.0% at 15 mg ml^{-1} and maintained the plateau of 76.7–77.2% at 20–30 mg ml^{-1} (Fig. 3). The methanolic extract from green leaves showed a better chelating effect than those from yellow fallen and red fallen leaves. In addition, chelating effects of extracts from yellow fallen and red fallen leaves were relatively parallel and increased from 18.2–26.0% at 5 mg ml^{-1} to 48.3–48.6% at 30 mg ml^{-1}. However, at 1 mg ml^{-1}, the chelating ability of EDTA was 99.5%. Apparently, three methanolic extracts from *T. catappa* leaves could chelate ferrous ions but were not as effective chelators as EDTA. Since ferrous ions are the most effective prooxidants in food systems (Yamaguchi, Tatsumi, Kato, & Yoshimitsu, 1988), the moderate to high chelating effects of methanolic extracts from *T. catappa* leaves would be beneficial.

Ko (1998) found that water extracts from green, yellow fallen and red fallen leaves scavenged ferrous ions by 61.1, 42.5 and 34.2% at 0.5 mg ml^{-1} and 93.7, 80.1 and 54.4% at 3 mg ml^{-1}, respectively. Obviously, with regard to chelating ability of ferrous ions, water extracts were better than methanolic extracts.

Both the scavenging ability on hydroxyl radicals and the antioxidant activity in the inhibition of linoleic acid peroxidation were found in the three methanolic extracts from *T. catappa* leaves. These results were different from those of Ogata, Hoshi, Shimotohno, Urano, and Endo (1997) that phenolic compounds, such as epigallocatechin gallate, epigallocatechin, baicalein,

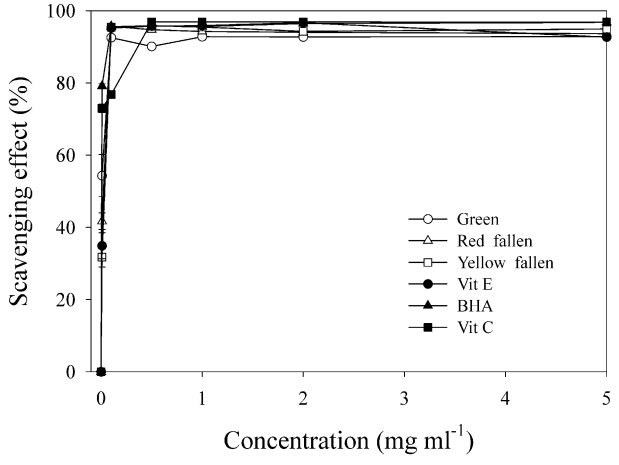

Fig. 2. Scavenging effect of methanolic extracts from *Terminalia catappa* leaves on 1,1-diphenyl-2-picrylhydrazyl radicals. Each value is expressed as mean±standard deviation ($n=3$).

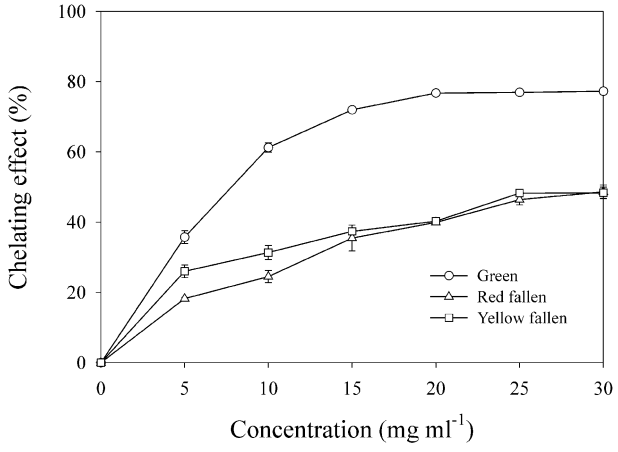

Fig. 3. Chelating effect of methanolic extracts from *Terminalia catappa* leaves on ferrous ions. Each value is expressed as mean±standard deviation ($n=3$).

catechin, baicalin and palmatine chloride, possessed the ability to inhibit lipid peroxidation but showed no scavenging effect on hydroxyl radicals. Although it was not clearly understood by which components in the three methanolic extracts the hydroxyl radicals were scavenged, some phenolic compounds described earlier were not responsible for this scavenging effect. To study the antioxidant mechanisms by some other potential antioxidant components, the fractionation of the methanolic extract and further identification are in progress.

Overall, for the three different leaves, the antioxidant activities in the DETBA method were in the order methanol > ethyl acetate > dichloromethane > pentane extracts. The antioxidant activities of extracts all showed a parabolic-like curve with a maximum at 0.1–0.5 mg. Reducing powers of the three methanolic extracts were excellent at 0.5 mg ml^{-1}. Scavenging effects of the three methanolic extracts on DPPH radicals were excellent at 0.1 mg ml^{-1}. At 30 mg ml^{-1}, chelating effects of methanolic extracts from green, yellow fallen and red fallen leaves on ferrous ions were 77.3, 48.6 and 48.3%, respectively. Although the three methanolic extracts could chelate ferrous ions, they were not as effective chelators as EDTA.

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