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Antioxidant Activity of Polyphenolic Compounds from *Dalbergia odorifera* T. Chen

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Abstract: Seven compounds were isolated from *Dalbergia odorifera* T. Chen and their antioxidant activities were studied with Oil Stability Index (OSI) method, reducing power and radical scavenging methods. The compounds were identified by spectroscopic methods as (1) pinocembrin (2) biochanin A (3) sativanone (4) biochanin B (5) naringenin (6) 3´-hydroxymelanettin and (7) eriodictoyl. Results showed that compounds 6 and 7 exhibited stronger antioxidant activity than commonly used synthetic antioxidant BHT in the presented study.

Key words: *Dalbergia odorifera*, antioxidant activity, oil stability index, reducing power, radical scavenging activity

INTRODUCTION

Lipid oxidation not only produces off-taste of food but also decreases safety and nutritional quality of foods. In addition, *in vivo* lipid peroxidation may lead to aging, heart disease and cancer (Frankel, 1996; Tsuda *et al*., 1994). Oxidation of lipid-containing foods can be retarded by adding effective antioxidants, which will lengthen the shelf life of foods (Guo *et al*., 2005).

Synthetic antioxidants, such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT) and Tert-Butylhydro-Quinone (TBHQ) are widely used in the food industry, however, their safety have been a concern to consumers (Ames, 1983; Baardseth, 1989; Wichi, 1988). Therefore, development and utilization of antioxidants from natural origin are desired (Guo *et al*., 2005; Namiki, 1990).

Dalbergia odorifera T. Chen (*D. odorifera*) has been used traditionally as medicine in China and recommended for the treatment of the epigastria and abdominal pain, costal and hypochondriac pain, traumatic injuries and the bleeding from lacerations. It was reported that this plant contains mainly phenolic components (Goda *et al*., 1992; Ogata *et al*., 1990; Yahara *et al*., 1989).

The antioxidant activity of *D. odorifera* has been reported in literatures (Wang *et al*., 2000; Weng *et al*., 1998; Yu *et al*., 2007). However, the antioxidant activity of *D. odorifera* was only evaluated with OSI method, more extensive information was required to comprehend the antioxidant activity of *D. odorifera*. Here we report the isolation, characterization and antioxidant activity of compounds isolated from this plant.

MATERIALS AND METHODS

Chemicals and materials: The commercially dried heartwood of *D. odorifera* was purchased from a traditional Chinese medicine company in Urumqi, China and identified by Dr Hai-hong Li in Xinjiang Hospital of Traditional Chinese Medicine, Urumqi, China. The heartwood of *D. odorifera* was further dried in a vacuum oven at 40°C under the pressure of 2 mmHg for 24 h. After cooled to ambient temperature, the dried heartwood was powdered with a grinder and sieved through a 60-mesh sieve. The powder was stored in brown flasks in the darkness before use.

2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Amresco Inc. (OH, USA). Silica gel (100 ~ 200, 200 ~ 300 mesh) was obtained from Qingdao Ocean Chemical Factory (Qingdao, China). Other chemicals used were of analytical grade and obtained from Shanghai Chemical Reagent Co. (Shanghai, China).

Preparation of lard: The lard was wet rendered from fresh pig fat purchased from Shanghai Slaughter House, filtered through three-layer cotton gauze, cooled to room temperature and stored in a deep-freezing refrigerator before use.

Extraction and isolation: The dried powdered heartwood of *D. odorifera* (1.5 kg) was extracted by a Soxhlet extractor with petroleum ether and ethyl acetate successively for 24 h. After the removal of the solvents under reduced pressure in a rotary evaporator, 10 g of petroleum ether extract and 70 g of ethyl acetate extract

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were obtained. Seventy grams of ethyl acetate extract was subjected to column chromatography on silica gel column (100 ~ 200 mesh) eluted with petroleum ether/ethyl acetate mixtures of increasing polarity (10: 1-1: 1). Fourteen fractions were obtained according to Thin Layer Chromatography (TLC) analysis using a mixture of petroleum ether/acetone and chloroform/acetone as the developing solvents. The spots on the TLC plates were detected with an UV detection lamp (365 nm) and colored with iodine. Fractions 1-7 were subjected to re-chromatograph on silica gel column (200 ~ 300 mesh).

Elucidation of the chemical structures: Mass spectra were obtained with an Agilent 5973N mass spectroscopic instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). Ultraviolet spectra (UV) were obtained with a Cary-100 spectroscopic instrument (Varian, Inc., Palo Alto, CA, USA). ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were obtained with a Bruker AV 500 MHZ NMR Spectrometer (Bruker Corporation, Karlsruhe, Germany).

Antioxidant assay

OSI method: American Oil Chemists' Society Official Oil Stability Index (OSI) method was used to determine the efficacy of compounds isolated from *D. odorifera* on relative autoxidation resistance of lard samples. An Omnion Oxidative Stability Instrument (Omnion, Inc., MA, USA) was used to determine the Induction Period (IP) of oxidation under the following conditions: temperature of 100°C; air flow rate of 20 L/h. BHT was used as positive control.

Reducing power method: The reducing power of the compounds isolated from *D. odorifera* was determined by the methods reported (Jayaprakasha *et al*., 2001). A methanolic solution (0.5 mL) of the samples at various concentrations was mixed with 2.5 mL of phosphate buffer solution (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%, w/v). The mixture was incubated at 50°C for 20 min. A portion of 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture and centrifuged at 3000 rpm for 10 min. Then 2.5 mL of supernatant was mixed with 2.5 mL of deionized water and 0.5 mL of ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm. The increase in the absorbance of reaction mixture indicates the increase of reducing power of samples. BHT was assayed at the same concentration as positive control.

$ABTS^{\bullet+}$ scavenging method: The ABTS radical scavenging activity of the isolated compounds was determined by the methods reported by Re *et al.* (1999) with some modifications. ABTS was dissolved in water to 7 mM concentration. $ABTS^{\bullet+}$ was generated by reacting ABTS stock solution with potassium persulfate (2.45 mM, final concentration) and the mixtures stood in the dark at room temperature for 16 h before used. The $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C for 30 min. A methanolic solution (0.2 mL) of the samples at various concentrations was mixed with 2.0 mL of diluted $ABTS^{\bullet+}$ solution. After reacting at ambient temperature for 20 min, the absorbance was measured at 734 nm. The decline of the absorbance of the reaction mixture indicates the increase of $ABTS^{\bullet+}$ scavenging activity. BHT was used as positive control. The scavenging activity of the $ABTS^{\bullet+}$ was calculated using the formula given below:

$$ABTS^{\bullet+} \text{ scavenging activity } (\%) = [1-(S-S_b)/(C-C_b)] \times 100\%$$

S: Absorbance of the sample; S_b: Absorbance of the blank sample (2.0 mL of methanol plus 0.2 mL of sample solution containing different concentrations); C: Absorbance of the control (2.0 mL of $ABTS^{\bullet+}$ solution plus 0.2 mL of methanol); C_b: Absorbance of the blank control (methanol).

Statistical analysis: The data were expressed as means±standard error of three replicates. Analysis of variance and significant differences among means were tested by one-way Analysis of Variance (ANOVA), using SPSS (Version 17.0, SPSS Inc., Chicago, IL, USA). The $p<0.05$ was regarded as significant and $p<0.01$ as very significant.

RESULTS AND DISCUSSION

Elucidation of the chemical structures: The spectrometric data for seven compounds isolated from *D. odorifera* were listed in Table 1. Their chemical structures shown in Fig. 1 was confirmed by integrally analyzing UV, EI-MS, ^{1}H and ^{13}C-NMR data obtained and compared with the data published.

Compound 1 (157mg) was isolated on silica gel column with petroleum ether/acetone (10: 1-10: 2) as white powder from the fraction two. It was determined to be 5,7-dihydroxy-2-phenylflavanone (pinocembrin) by comparing the data reported (Kuroyanagi *et al*., 1983).

Compounds 2 (98 mg) and 3 (367 mg) were both obtained from fraction three on silica gel with petroleum ether/ethyl acetate (8: 1-3: 1). Compound 2 was colorless needle crystals. Its ^{1}H NMR, ^{13}C NMR and EI-MS data were in good accordance with the data for 4´-methoxy-5,7- dihydroxyisoflavone (biochanin A) in literatures reported (Hedin and Phillips, 1992).

Compound 3, colorless plate-like crystals, showed $[M]^{+}$ peak at m/z = 300. Its ^{1}H NMR, ^{13}C-NMR and EI-MS were in agreement with the literature data for 7-hydroxy-2´4´-dimethoxyisoflavanone (sativanone) reported (Jain and Nayyar, 1987).

Table 1: Spectral data of the compounds isolated from *D. odorifera*

Compound	Data of spectra			
	^{1}H NMR (500 MHz)	^{13}C NMR (125 MHz)	EI-MS (70eV, m/z)	UV (nm, Ethanol)
1	12.19 (1H, s), 9.65 (1H, s), 7.44 (5H, m), 6.02 (1H, d), 6.00 (1H, d), 5.53 (1H, dd), 3.14 (1H, dd), 2.79 (1H, dd) (acetone-d_6)	196.70, 167.24, 165.19, 164.04, 139.88, 129.41, 129.34, 127.20, 103.17, 96.95, 95.88, 79.84, 43.50 (acetone-d_6)	256 (100), 255 (59), 238 (9), 179 (73), 153 (17), 152 (61), 124 (34), 104 (12), 103 (13), 96 (11), 78 (12), 77 (11), 69 (13), 51 (6)	324, 290
2	13.01 (1H, s), 9.73 (1H, s), 8.17 (1H, s), 7.53 (2H, d), 6.98 (2H, d), 6.41 (1H, d), 6.28 (1H, d), 3.83 (3H, s) (acetone-d_6)	180.53, 164.00, 162.88, 159.63, 158.00, 153.48, 130.07, 123.15, 122.75, 113.48, 105.13, 98.86, 93.50, 54.56 (acetone-d_6)	285 (20), 284 (100), 283 (18), 269 (14), 152 (10), 142 (5), 133 (7), 132 (30), 124 (6), 117 (6), 89 (8)	330, 262
3	7.60 (1H, d), 6.99 (1H, d), 6.55 (1H, d), 6.50 (1H, dd), 6.47 (1H, dd), 6.33 (1H, d), 4.53 (1H, dd), 4.39 (1H, dd), 4.15 (1H, dd), 3.77 (3H, s), 3.75 (3H, s) (MeOD-d_4)	194.37, 166.41, 165.76, 162.18, 159.85, 132.04, 130.37, 117.32, 115.64, 111.70, 105.93, 103.61, 99.91, 72.01, 55.96, 55.79, 48.90 (MeOD-d_4)	300 (18), 165 (11), 164 (100), 149 (51), 133 (7), 132 (30), 124 (6), 121 (27), 117 (6), 89 (8)	311, 276
4	9.63 (7-OH), 8.18 (1H, s), 8.07 (1H, d), 7.56 (2H, d), 7.013 (2H, d), 6.98 (1H, dd), 6.91 (1H, d), 3.83 (3H, s) (acetone-d_6)	175.61, 163.2, 160.46, 158.81, 153.4, 131.01, 128.51, 125.55, 124.97, 118.61, 115.69, 114.38, 103.21, 55.55 (acetone-d_6)	269 (17), 268 (100), 267 (38), 255 (6), 253 (18), 134 (4), 133 (6), 132 (46), 117 (9), 89 (10)	302, 249
5	7.31 (2H, d), 6.81 (2H, d), 5.89 (1H, d), 5.88 (1H, d), 5.33 (1H, d), 3.10 (1H, dd), 2.69 (1H, dd) (MeOD-d_4)	197.78, 168.33, 165.46, 164.88, 159.02, 131.07, 129.04, 116.31, 103.34, 97.03, 96.15, 80.47, 44.03 (MeOD-d_4)	272 (73), 271 (46), 179 (30), 166 (30), 153 (100), 120 (70)	326, 289, 224
6	7.06 (1H, s), 7.02 (1H, s), 6.93 (1H, d), 6.92 (1H, d), 6.85 (1H, dd), 6.13 (1H, s), 3.97 (3H, s) (MeOD-d_4)	164.28, 158.42, 153.40, 150.42, 148.40, 146.78, 145.00, 128.44, 121.52, 116.65, 116.61, 113.25, 112.20, 111.27, 101.02, 56.84 (MeOD-d_4)	301 (19), 300 (100), 273 (10), 272 (58), 258 (12), 257 (68), 187 (6), 155 (6), 136 (12), 115 (12), 113 (8), 69 (18)	344, 300, 256, 233
7	6.91 (1H, s), 6.78 (2H, m), 5.89 (1H, d), 5.88 (1H, d), 5.26 (1H, dd), 3.06 (1H, dd), 2.68 (1H, dd) (MeOD-d_4)	197.75, 168.31, 165.42, 146.85, 146.47, 131.74, 114.68,105.93, 105.93, 80.47, 44.07 (MeOD-d_4)	288 (69), 287 (33), 179 (29), 166 (43), 153 (100), 136 (52), 137 (34), 123 (26)	324, 288

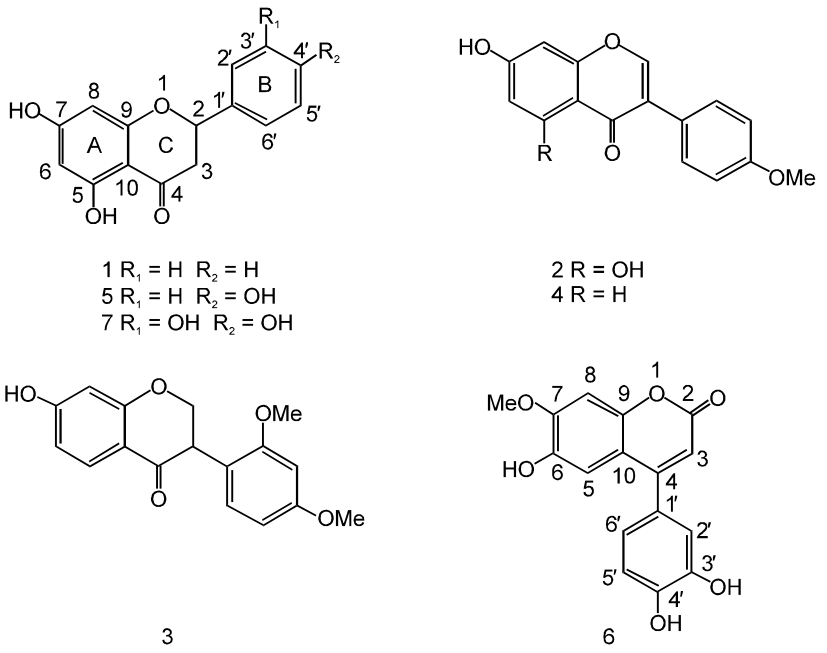

Fig. 1: The structures of the compounds isolated from *D. odorifera*

Compound 4 (31 mg), white needle crystals, was isolated from fraction four on silica gel chromatography column with petroleum ether/acetone (1: 1). The ^{1}H-NMR, ^{13}C NMR and EI-MS data of compound 4 agreed with those reported for 7-hydroxy-4'-methoxyisoflavone (biochanin B) (Herath *et al*., 1998; Zhao *et al*., 2009).

Compound 5 (48 mg), isolated from fraction five with petroleum ether/acetone (1: 1-1: 2) as white needle crystals, had a similar NMR data with compound 1. The UV, ^{1}H-NMR, ^{13}C NMR and EI-MS data of compound 5 agreed with the data for 4',5,7-trihydroxyflavanone (naringenin) (Hedin and Phillips, 1992; Olsen *et al*., 2008).

Both compound 6 (15 mg) and compound 7 (10 mg) were isolated from fraction seven with chloroform/acetone (1: 0-1: 1) on silica gel chromatography column. Compound 6 was a white powder. Its ^{1}H-NMR, ^{13}C NMR and EI-MS data agreed with 3',4',6-trihydroxy-7-methoxyneoflavone (3'-hydroxymelanettin) in the literature (Chan *et al*., 1997).

Compound 7, a light yellow powder, also showed similar NMR data with compounds 1 and 5. Its ^{1}H-NMR, ^{13}C NMR and EI-MS data were in good accordance with 3´,4´,5,7-tetrahydroxyflavanone (eriodictoyl) reported (Encarnacion *et al*., 1999; Hedin and Phillips, 1992).

Antioxidant activity assayed by OSI method: The antioxidant activity of the samples on retarding the lard oxidation was expressed as the Protection factor (Pf), which was calculated using the following formula:

$$Pf = \frac{IP_{antioxidant}}{IP_{lard}}$$

The $IP_{antioxidant}$ and IP_{lard} were the oxidation Induction Period (IP) of lard with and without antioxidant, respectively. According to report by Weng and Wu (2000), a higher value of Pf means a greater antioxidant activity of a sample. If Pf<1, the sample had pro-oxidant activity; if Pf = 1, the sample had no antioxidant activity; if 2≤Pf>1, the sample had antioxidant activity; if 3≤Pf>2, the sample had an obvious antioxidant activity and if Pf>3, the sample had a strong antioxidant activity.

As shown in Table 2, all the compounds isolated from *D. odorifera* presented antioxidant activity against lard oxidation at the concentrations of 0.012 and 0.02%. Among them, compounds 6 and 7 presented stronger antioxidant activity than commonly used synthetic antioxidant BHT. The Pf values of compound 7 are double the values of BHT at the concentrations of 0.012 and 0.02%.

Antioxidant activity assayed by reducing power method: Some previous studies have reported that the antioxidant activity was concomitant with the reducing power (Jeong *et al*., 2004). We investigated the reducing power of the compounds isolated from *D. odorifera*. Figure 2 showed the reducing powers of the compounds isolated from *D. odorifera* and BHT by using the potassium ferricyanide reduction method. A strong reducing power was noted for compounds 6 and 7. The result showed that the reducing power of test samples decreased in the following order: compounds 6 - 7 > BHT > compounds 1 - 5.

Table 2: The antioxidant Protection factors (Pfs) of compounds isolated from *D. odorifera* determined using OSI method[a]

Concentration (w/w) (%)	Compounds 1	2	3	4	5	6	7	BHT
0.012	1.09±0.10	1.14±0.01	1.03±0.01	1.20±0.02	1.13±0.02	4.20±0.02	6.48±0.31[b**]	3.61±0.10
0.02	1.20±0.51	1.28±0.08	1.15±0.02	1.27±0.05	1.24±0.05	5.57±0.07[b*]	9.32±0.28[b**]	4.22±0.48

[a]Data are means±standard error of three replicates, Lard: 3.00±0.02 g.
[b]*p<0.05, vs BHT; **P<0.01, vs BHT

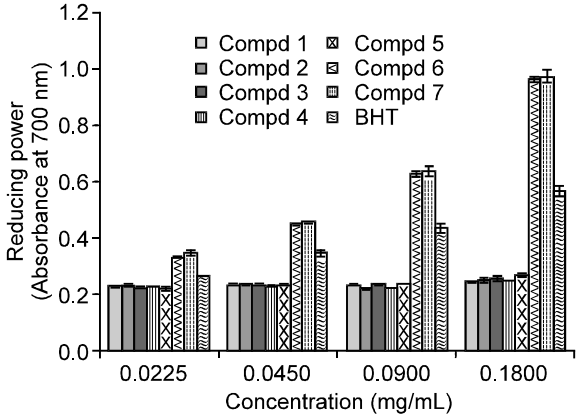

Fig. 2: Reducing power (absorbance at 700 nm) of compounds isolated from *D. odorifera* and BHT. Each vertical bar represents the standard deviation for data point (n = 3)

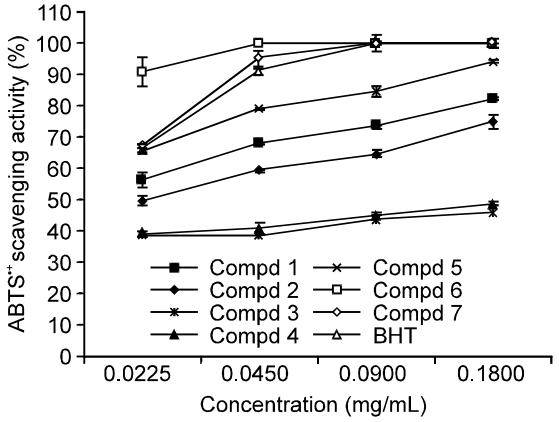

Fig. 3: $ABTS^{\bullet+}$ scavenging activity (%) of compounds isolated from *D. odorifera* and BHT. Each vertical bar represents the standard deviation for data point (n = 3)

Antioxidant activity assayed by $ABTS^{\bullet+}$ method: The antioxidant activity of compounds isolated from *D. odorifera* was measured by the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. As shown in the Fig. 3, compounds 6 and 7 had stronger antioxidant activity than that of BHT. The free-radical scavenging abilities of test samples decreased in the following order: compound 6 > compound 7 > BHT > compound 5 > compound 1 > compound 2 > compounds 3 - 4. Except compound 6, which is a neoflavonoids, the other isolated compounds are flavonoids. Many flavonoids were reported to have antioxidant activities (Rice-Evans *et al*., 1996; Seyoum *et al*., 2006). Among flavonoids we isolated, compounds 6 and 7 possess ortho-dihydroxyl groups in the B-ring, which result in higher antioxidant activities of the two compounds (Chen *et al*., 2002; Rice-Evans *et al*., 1996). As a result, compounds 6 and 7 had the strongest activity among the seven compounds and BHT. Additionally, the antioxidant activity between compounds 6 and 7 had a little difference determined by using different methods. In antioxidant assay by using OSI method, compound 7 had a little stronger activity than that of compound 6. But in the other two antioxidant assay methods, compound 6 exhibited equivalent antioxidant activity compared with compound 7 determined by using reducing power method and a little stronger antioxidant activity tested by using $ABTS^{\bullet+}$ method. This might result from different testing mechanism for each method. In antioxidant assay using OSI method, compound 7 had a little stronger activity than that of compound 6. Compound 7 performed the strongest action not only for possessing *ortho*-dihydroxyl groups, but also for the chelating ability of the structure between 5-hydroxyl in the A-ring and 4-oxo function in the C-ring, which might eliminate the catalytic oxidation performance of the trace amounts of metal ions (Mira *et al*., 2002). The metal ion chelating power may strengthen the antioxidant activity of compound 7 in lard, especially when trace transition metal ions such as ferric ion and copper ion occur. Compound 6, possessing no chelating ability because of lack of 4-oxo function, exhibited a little weaker antioxidant activity than that of compound 7 with OSI method in lard. The further study showed that when there was 1.5 ppm Fe^{3+} in lard, it showed a clearly pro-oxidation activity for the Pf decreased to 0.98. The existence of 1.5 ppm Fe^{3+} had a remarkable effect on the protection factor of 0.01% compound 6 in lard, for the Pf decreased by 1.6 from 3.94 to 2.34. The same concentration of Fe^{3+} had little effect on the protection factor of 0.01% compound 7 in lard because the Pf changed only 0.29 (see Table 3). In other words, compound 7 had much stronger ability to eliminate the catalytic oxidation performance of ferric ion than compound 6. This difference might be due to the structure of 5-hydroxyl in the A-ring and 4-oxo function in the C-ring of compound 7, which could exert chelating ability to metal ions.

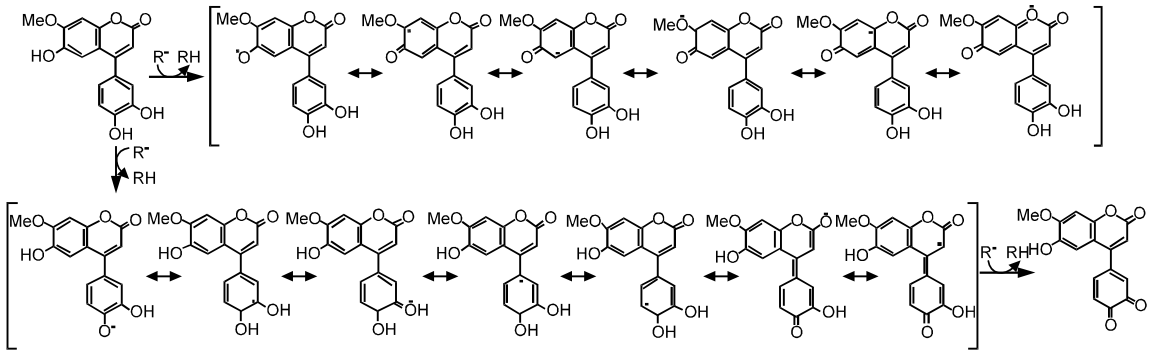

Fig. 4: Explanation of why compound 6 possesses strong antioxidant activity

Table 3: The Protection factor (Pf) of compounds 6 and 7 with citric acid and Fe^{3+} determined using OSI method[a]

Component in Lard	Pf
0.01% Citric acid	1.07±0.005
0.01% Compound 6	3.94±0.006
0.01% Compound 7	5.39±0.012
0.01% Citric acid + 0.01% Compound 6	4.46±0.003
0.01% Citric acid + 0.01% Compound 7	5.66±0.015
1.5 ppm Fe^{3+}	0.92±0.005
1.5 ppm Fe^{3+} + 0.01% Compound 6	2.34±0.006
1.5 ppm Fe^{3+} + 0.01% Compound 7	5.10±0.005

[a]Data are means±standard error of three replicates, Lard: 3.00±0.02 g

Further comparing the structures of compounds 6 and 7, two hydroxyl groups on A-ring of compound 7 are very difficult to provide hydrogen atoms to free radicals because both of hydroxyl groups locate on para- or ortho-positions of carbonyl group (Fig. 1) which possesses strong electron withdrawing effect and meta-oxyl groups which also have some electron withdrawing effect (Duan *et al*., 1998). On the other hand, compound 6 can donate more hydrogen atoms than compound 7 because a hydroxyl group on A-ring can act as a good hydrogen providing group as it has an ortho-methoxyl group which has strong electron-donating effect, a para intramolecular ester group which bonds the para-position via oxygen atom and also has weak electron-donating effect and no strong electron-withdrawing group on both of ortho- or para-positions of the hydroxyl group. Compound 6 possess an unsaturated bond in the C-ring allowed electron delocalization across the molecule for stabilization of the aryloxyl radical (Fig. 4), this may increase H-providing ability of hydroxyl groups both on A-ring and B-ring. In reducing power and $ABTS^{\bullet+}$-scavenging power experiments, water, which can effectively inactivate transition metal cations, is used as a media, so metal-chelating ability is not important in the antioxidant compounds. As a result, compound 6 demonstrate stronger antioxidant power than compound 7 in these cases.

Above analysis explains quite clearly why compound 7 is a stronger antioxidant in lard by OSI method, but a

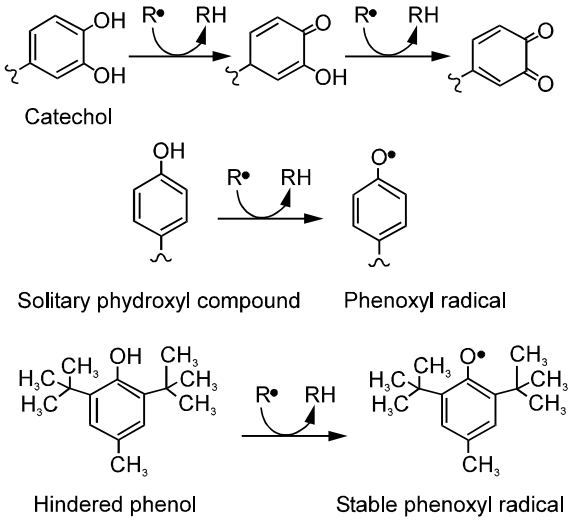

Fig. 5: Explanation of why compounds 6, 7 and BHT possess strong antioxidant activities

weaker antioxidant by reducing power method and $ABTS^{\bullet+}$ scavenging method than compound 6.

Compounds 1~5 and BHT presented less antioxidant activities than those of compounds 6 and 7. This is mainly due to the less numbers of hydroxyl groups, especially no ortho-dihydroxyl groups in B-ring (Fig. 1 and 5). On the other hand, the synthesis antioxidant BHT possess two large space hindrance tert-butyl groups at the adjacent positions, which made BHT easy to loss hydrogen and form a more stable free radical than compounds 1~5 (Fig. 5).

D. odorifera was found to have obvious antioxidant activity (Wang *et al*., 2000; Weng *et al*., 1998; Yu *et al*., 2007). However, the compounds from this herb in this paper were totally different from the previous literature (Wang *et al*., 2000; Yu *et al*., 2007). This paper revealed additional information on compounds with antioxidant activities from *D. odorifera* and suggested that this herb may be a promising source of natural antioxidants for food.

Conclusion: In conclusion, the results of this study demonstrated that some compounds from *D. odorifera* had strong antioxidant activity and suggested that this medicinal herb may be a promising source of natural antioxidants.

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