Isolation of Some Compounds from Nutmeg and their Antioxidant Activities

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Abstract

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Six compounds, licarin-B (1), dehydrodiisoeugenol (2), malabaricone B (3), malabaricone C (4), β-sitosterol (5), and daucosterol (6) were isolated from the seed (nutmeg) of *Myristica fragrans* Houtt and identified. Among them, malabaricone B was identified for the first time in nutmeg. Antioxidant activities of the isolated compounds were studied using oil stability index (OSI), reducing power, $ABTS^{\bullet+}$ scavenging, and $DPPH^{\bullet}$ scavenging methods. The results showed that Malabaricone C is an efficient antioxidant agent which exhibits a stronger antioxidant activity than the commonly used synthetic antioxidants in all studied methods. This compound may have a potential to be used as a natural antioxidant in food.

Keywords: malabaricone C; oil stability index; reducing power; radical scavenging activity

Lipid peroxidation is one of the major factors which produce food deterioration during processing and storage (St. Angelo *et al*. 1996). In addition, *in vivo* lipid peroxidation causes ageing, heart diseases, and carcinogenesis (Tsuda *et al*. 1994; Frankel 1996). Antioxidants are often used in oils and fatty foods to retard their autoxidation and enhance their shelf life. Many spices and herbs have been found to possess a potent antioxidant activity and can be rich sources of natural antioxidants (Su *et al*. 1986; Weng *et al*. 1998).

Myristica fragrans Houtt., an aromatic evergreen tree, is named as RouDouKou in Chinese traditional medicine. The seed (nutmeg) of *M. fragrans* is widely used for spice and medicinal purposes (Ozaki *et al*. 1989; Capasso *et al*. 2000). Nutmeg can be kept for a very long time as spice without lipid peroxidation. Therefore, it may be a good source of natural antioxidants. The antioxidant activity of nutmeg powder in lard has been reported by our group (Weng *et al*. 1998), however, according to the best of our knowledge, the identification of the components from nutmeg is not complete no reports exist on detailed evaluation of the antioxidant activity and antioxidant components of nutmeg. Therefore, the aim of this paper was to investigate the components of nutmeg and their antioxidant activity.

MATERIAL AND METHODS

Chemicals and material. Nutmeg was purchased from a traditional Chinese herbal company in Urumqi, China and identified by Dr. Hai-hong Li in Xinjiang Hospital of Traditional Chinese Medicine, Urumqi, China. The herb was further dried at 40°C under the pressure of 2 mm Hg for 24 hours. After being powdered with a grinder and sieved through a 60-mesh sieve, the resulting nutmeg powder was stored at –20°C.

2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Amresco Inc. (Cleveland, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) was purchased from Sigma-Aldrich (St. Louis, USA). Silica gel (100–200 mesh and 200–300 mesh) was purchased from Qingdao Ocean Chemical Factory (Qingdao, China). Other chemicals used in the experiment were of analytical grade and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of lard. The lard was obtained wet from fresh pig fat tissues, and was filtered through three-layer cotton gauze. After cooling to room temperature, it was stored deep-frozen until required.

Extraction and isolation. Two kg of nutmeg powder was extracted in a Soxhlet extractor successively with petroleum ether, ethyl acetate, acetone, and ethanol, with each of these for 20 hours. After the solvents removal under vacuum, 1082 g of petroleum ether extract, 50 g of ethyl acetate extract, 14 g of acetone extract, and 62 g of methanol extract were obtained, respectively. Antioxidant activities of the four extracts were assessed with OSI method, ethyl acetate extract having been found to possess the highest antioxidant activity. This extract was subjected to chromatographic separation.

Forty-five grams of ethyl acetate extract was separated by column chromatography on a silica gel column (100–200 mesh) eluted with petroleum ether/ethyl acetate mixtures of increasing polarity (10:1–1:10). Five 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 coloured with iodine. The five fractions were subjected to re-chromatography on a silica gel column (200 to 300 mesh) to give six compounds (1–6).

OSI method. The antioxidant activity of the samples (nutmeg powder, extracts of nutmeg, extract residues, and the compounds isolated from nutmeg) was tested on an Oxidative Stability Instrument (Omnion, Inc., Rockland, USA) in 5 ± 0.02 g lard at 100°C. The air flow rate was fixed at 20 l/hour. BHT was used as a positive control. The antioxidant activity of the samples manifested by retarding the lard oxidation was expressed as the protection factor (*Pf*), which was calculated according to the following formula:

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

where:

$IP_{\text{antioxidant}}$, IP_{lard} – oxidation induction periods (IP) of lard with and without antioxidant

Reducing power method. The reducing power of the compounds isolated from nutmeg was measured by the methods described in the literature (Jayaprakasha *et al*. 2001). A methanolic solution (0.5 ml) of each of the test samples (0.05 and 0.10 mg/ml) was mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%, w/v) solution. After incubation at 50°C for 20 min, a portion of trichloroacetic acid (2.5 ml, 10%, w/v) was added and the resulting mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant (2.5 ml) was mixed with deionised water (2.5 ml) and ferric chloride (0.5 ml, 0.1%, w/v), and the absorbance was measured at 700 nm. BHT was assayed at the same concentration as the positive control.

ABTS$^{\bullet+}$ scavenging method. The DPPH$^{\bullet}$ scavenging activity of the isolated compounds was carried out as reported in the literature (Re *et al*. 1999) with some modifications. Briefly, ABTS$^{\bullet+}$ was generated by reacting ABTS solution (7mM) with potassium persulphate (2.45mM, final concentration) for 14 h in the dark at room temperature. Then, the ABTS$^{\bullet+}$ solution was diluted with methanol to obtain the absorbance of 0.70 (± 0.02) at 734 nm and further equilibrated at 30°C for 30 minutes. Methanolic solution (0.2 ml, 0.02 and 0.04 mg/ml) of each sample was mixed with diluted ABTS$^{\bullet+}$ solution (2.0 ml). The absorbance of the reaction mixture was measured at 734 nm after the reaction at ambient temperature for 20 minutes. BHT was used as positive control. The scavenging activity of ABTS$^{\bullet+}$ was calculated according to the expression given below:

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

where:

S – absorbance of the sample

S_b – absorbance of the blank sample (2.0 ml of methanol plus 0.2 ml of sample solution)

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)

DPPH• scavenging method. The DPPH• scavenging activity of the isolated compounds was determined as reported in the literature (Pyo *et al.* 2004) with minor modifications. A methanolic solution (0.2 ml) of each of the test samples (0.02, 0.04, and 0.08 mg/ml) was added to DPPH• methanolic solution (2.0 ml, 1×10^{-4}M). The absorbance at 517 nm was measured after the mixture was allowed to stand in the dark for 60 minutes. BHT was assayed at the same concentration as the positive control. The scavenging activity of DPPH• was calculated according to the Equation (1) where: S – absorbance of the sample; S_b – absorbance of the blank sample (2.0 ml of methanol plus 0.2 ml of sample solution); C – absorbance of the control (2.0 ml of DPPH• 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. The data were subjected to the analysis of variance and significant differences between the means to one way analysis of variance (ANOVA), using SPSS (Vers. 17.0, SPSS Inc., Chicago, USA). $P < 0.05$ was regarded as significant and $P < 0.01$ as very significant.

RESULTS AND DISCUSSION

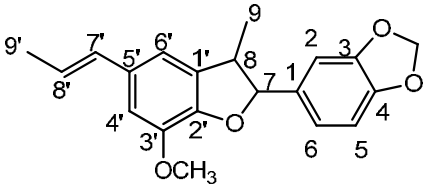
Elucidation of the chemical structures

Mass spectra were obtained with Agilent 5973N mass spectroscopic instrument (Agilent Technologies, Inc., Santa Clara, USA). Nuclear magnetic resonance (NMR) spectra were determined with a Bruker AV 500 MHz NMR spectrometer (Bruker Corporation, Karlsruhe, Germany). Their chemical structures shown in Figure 1 were confirmed by analysing ^{1}H, ^{13}C-NMR, and MS data obtained and comparing them with the data published.

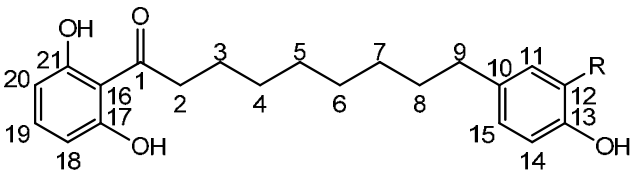
Compound 1 (312 mg) was isolated as white crystals (petroleum ether). The molecular formula was determined to be $C_{20}H_{20}O_4$ by EI-MS ($[M]^+$ at *m/z* 324). Based on the comparison of the nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) data with the reported values, it was identified as licarin-B (Shin *et al*. 1988; Lu *et al*. 2005).

Compound 1: ^{1}H-NMR ($CDCl_3$-d_6, 500 MHz) δ: 1.378 (3H, d, J = 6.8 Hz, H-9), 1.870 (3H, d, J = 6.3 Hz, H-9'), 3.419 (1H, dd, J = 15.3Hz, 8.0 Hz, H-8), 3.890 (3H, s, OCH_3-3'), 5.095 (1H, d, J = 9.0Hz, H-7), 5.950 (2H, s, -OCH_2O-), 6.114 (1H, m), 6.358 (1H, d, J = 15.6Hz, H-7'), 6.768 (1H, d, J = 7.2Hz, H-6'), 6.770 (1H, d, J = 6.0 Hz, H-5), 6.791 (1H, d, J = 6.0 Hz, H-5), 6.878 (1H, d, J = 7.8 Hz, H-6), 6.928 (1H, s); ^{13}C-NMR ($CDCl_3$-d_6, 500 MHz) δ: 147.85 (C-3), 147.56 (C-4), 146.45 (C-2'), 144.08 (C-3'), 134.28 (C-6'), 133.04 (C-1'), 132.18 (C-5'), 130.88 (C-7'), 123.45 (C-8'), 120.28 (C-6), 109.19 (C-4'), 108.02 (C-5), 106.75 (C-2), 101.05 (-OCH_2O-), 93.39 (C-7), 55.90 (-OCH_3-3'), 45.75 (C-8)18.36 (C-9'), 17.85 (C-9); EI-MS *m/z* (rel. int. %) 324 (100), 309 (14), 278 (36), 202 (19), 189 (28), 135 (60), 77 (16).

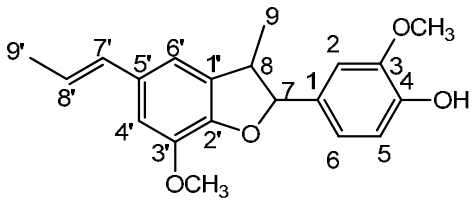
Compound 2 (75 mg) was also isolated as white crystals (petroleum–acetone). Its molecular ion peak ($[M]^+$ *m/z*) was 326. Both its ^{1}H NMR and



1



3 R = H
4 R = OH



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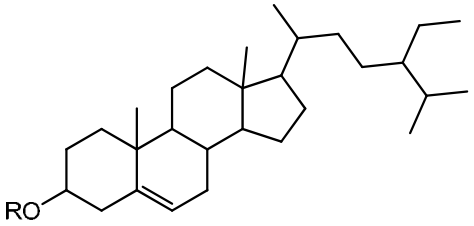

5 R = H
6 R = glc

Figure 1. The structures of the compounds isolated from nutmeg

^{13}C NMR were similar to those of compound 1 and are in good accordance with the literature reported data for dehydrodiisoeugenol (Forrest *et al*. 1973; Shin *et a*l. 1988).

Compound 2: ^{1}H-NMR ($CDCl_3$-d_6, 500 MHz) δ: 1.373 (3H, dd, J = 6.9 Hz, 2.4 Hz, H-9), 1.869 (3H, dd, J = 1.5 Hz, 6.6 Hz, H-9'), 3.447 (1H, dd, J = 9.4Hz, 6.6Hz, H-8), 3.876 (6H, s, -OCH_3-3, -OCH_3-3'), 5.099 (1H, d, J = 8.4Hz, H-7), 5.627 (1H, s, OH-4), 6.104 (2H, m, H-8'), 6.363 (1H, dd, J = 15.6 Hz, 1.5 Hz, H-7'), 6.765 (1H, s, H-6'), 6.785 (1H, s, H-4'), 6.879 (1H, d, J = 8.4 Hz, H-5), 6.911 (1H, d, J = 8.4Hz, H-6), 6.971 (1H, s); ^{13}C-NMR ($CDCl_3$-d_6, 500 MHz) δ: 146.64 (C-3), 145.75 (C-4), 144.13 (C-3'), 133.24 (C-1'), 132.18 (C-5'), 132.06 (C-1), 130.90 (C-7'), 123.47 (C-8'), 119.95 (C-6), 114.03 (C-3'), 113.28 (C-6'), 109.20 (C-4'), 108.89 (C-2), 93.77 (C-7), 55.95 (CH3O-), 45.60 (C-8), 18.35 (C-9'), 17.53 (C-9); EI-MS *m/z* (rel. int. %) 326 (100), 311 (15), 309 (12), 295 (10), 202 (16), 189 (11), 151 (13), 137 (22), 91 (10).

Compound 3 (27 mg), white crystals (petroleum–acetone), showed $[M]^+$ peak at *m/z* = 342. Its structure was established by comparing NMR data with those reported for malabaricone B (Pham *et al*. 2000; Patro *et al*. 2005). This compound was previously isolated from mace but this is the first time it has been isolated from nutmeg.

Compound 3: ^{1}H-NMR (Acetone-d_6, 500 MHz) δ: 1.296 (8H, br.s, -CH_2-4,5,6,7), 1.518 (2H, m, -CH_2-3), 1.651 (2H, m, -CH_2-8), 2.462 (2H, t, J = 8Hz, -CH_2-9), 3.113 (2H, m, -CH_2-2), 6.383 (2H, d, J = 8Hz, H-18,20), 6.699 (2H, d, J = 8.5Hz, H-11,15), 6.969 (2H, d, J = 8.5Hz, H-12,14), 7.212 (1H, t, J = 8Hz, 8.5Hz, H-19), 7.997 (1H, s, -OH-13), 11.380 (2H, s, -OH-16,21); ^{13}C-NMR (Acetone-d_6, 500 MHz) δ: 208.78 (C = O-1), 163.02 (C-17, 21), 156.12 (C-13), 136.69 (C-19), 134.17 (C-10), 129.98 (C-11,15), 115.79 (C-12, 14), 110.87 (C-16), 108.36 (C-18,20), 45.27 (C-2), 35.58 (C-9), 32.61 (C-8), 29.95 (C-4), 29.64 (C-7), 29.49 (C-6), 29.34 (C-5), 25.13 (C-3); EI-MS *m/z* (rel. int. %) 342 (27), 324 (5), 232 (7), 204 (14), 165 (36), 152 (18), 137 (100), 120 (12), 107 (72).

Compound 4 (43 mg), light yellow crystals (petroleum–acetone), gave a similar NMR spectrum data as that obtained with compound 3. Its molecular formula was determined to be $C_{21}H_{26}O_5$ by EI-MS ($[M]^+$ at *m/z* 358) and was established as malabaricone C by comparing the NMR data with those reported (Pham *et al*. 2000; Patro *et al*. 2005).

Compound 4: ^{1}H-NMR (Acetone-d_6, 500 MHz) δ: 1.341 (8H, br.s, CH_2-4,5,6,7), 1.541 (2H, m, CH_2-8), 1.685 (2H, m, CH_2-3), 2.447 (2H, t, CH_2-9), 3.155 (2H, t, CH_2-2), 6.416 (2H, d, J = 8Hz, H-18,20), 6.507 (2H, dd, J = 2Hz, 8Hz, H-15), 6.674 (1H, d, J = 2Hz, H-11), 6.709 (1H, d, J = 8Hz, H-14), 7.244 (1H, t, J = 8Hz, H-19), 7.625 (1H, s, OH-13 or 12), 11.426 (2H, s, OH-16 or 21); ^{13}C-NMR (Acetone-d_6, 500 MHz) δ: 208.78 (C = O-1), 163.02 (C-16, 21), 156.12 (C-13), 136.69 (C-19), 134.17 (C-10), 129.98 (C-11), 115.79 (C-12, 14), 110.87 (C-16), 108.36 (C-18,20), 45.27 (C-2), 35.58 (C-9), 32.61 (C-8), 29.95 (C-4), 29.64 (C-7), 29.49 (C-6), 29.34 (C-5), 25.13 (C-3); EI-MS *m/z* (rel. int. %) 358 (38), 340 (2), 326 (21), 248 (39), 220 (17), 194 (13), 165 (27), 149 (14), 137 (100), 123 (72), 110 (78).

Compound 5 (13 mg), white needle crystals (acetone), showed $[M]^+$ peak at *m/z* = 414 and was identified with the standards of β-sitosterol with TLC using three different solvent systems.

β-sitosterol EI-MS *m/z* (rel. int. %) 415 (31), 414 (39), 396 (66), 381 (36), 329 (31), 303 (32), 275 (20), 255 (43), 213 (37), 161 (28), 145 (49), 121 (44), 105 (64), 81 (69), 69 (61), 57 (100).

Compound 6 (11 mg), white powder (methanol), showed MS spectra similar as compound 5 and was identified with the standards of daucosterol by TLC using three different solvent systems.

Daucosterol EI-MS *m/z* (rel. int. %) 414 (76), 396 (100), 381 (32), 329 (24), 303 (28), 275 (18), 255 (41), 213 (49), 161 (32), 145 (69), 121 (46), 105 (81), 81 (76), 55 (94).

Antioxidant activity assayed by OSI method

According to the reported literature (Weng & Wu 2000), a higher value of *Pf* means a greater antioxidant activity of the sample. If $Pf < 1$, the sample has pro-oxidant activity; if $Pf = 1$, the sample has no antioxidant activity; if $2 \geq Pf > 1$, the sample has antioxidant activity; if $3 \geq Pf > 2$, the sample has an obvious antioxidant activity; and if $Pf > 3$, the sample has a strong antioxidant activity. As shown in Table 1, among all the extracts, ethyl acetate extract showed a strong antioxidant activities, acetone extract showed an obvious antioxidant activity, methanol extract a weak antioxidant activity, and petroleum ether extract, however, showed a pro-oxidant activity. This means that nutmeg, especially its ethyl acetate extract, will be a potential source of antioxidants. The ethyl acetate extract was chromatographically separated and six compounds were isolated (see above).

Table 1. Antioxidant activities comparison of different solvents extracts, nutmeg powder, residue and isolated compounds

Group	*Pf*
Petroleum extract	0.88 ± 0.02
Ethyl acetate extract	7.00 ± 0.02
Acetone extract	2.82 ± 0.05
Methanol extract	1.08 ± 0.04
Compound 1	1.06 ± 0.01
Compound 2	1.32 ± 0.03
Compound 3	1.25 ± 0.23
Compound 4	7.55 ± 0.26
Compound 5	1.03 ± 0.07
Compound 6	1.09 ± 0.12
BHT	4.51 ± 0.19

IP of lard is 5.4 h; temperature was set at 100°C; air flow rate was 20 l/h, different solvents extracts were added 0.1% in lard, respectively; isolated compounds and BHT were added 0.02% in lard, respectively; data represent means of duplicate samples

All the isolated compounds were tested in the Oxidative Stability Instrument at 100°C in lard. Among them, compound 4 presented a stronger antioxidant activity than the synthetic antioxidant BHT at a concentration of 0.02%. Compounds 1, 5, and 6 hardly showed any antioxidant activity and compounds 2 and 3 showed only a weak antioxidant activity (Table 1).

Antioxidant activity assayed by reducing power method

Some previous studies reported that the antioxidant activity was concomitant with the reducing power (Jeong *et al.* 2004). The reducing power of the compounds isolated from nutmeg was investigated to evaluate their antioxidant potentials. It was noted that, as seen in Figure 2, compound 4 was the only one which showed a stronger reducing power than was that of BHT. As the concentration increased from 0.02 mg/ml to 0.1 mg/ml, the reducing power of compound 4 increased rapidly from 0.39 to 0.93 (absorbance at 700 nm). All other compounds demonstrated a weak antioxidant activity assessed with the reducing power method, for their reducing power changed little with the increasing concentration (Figure 2).

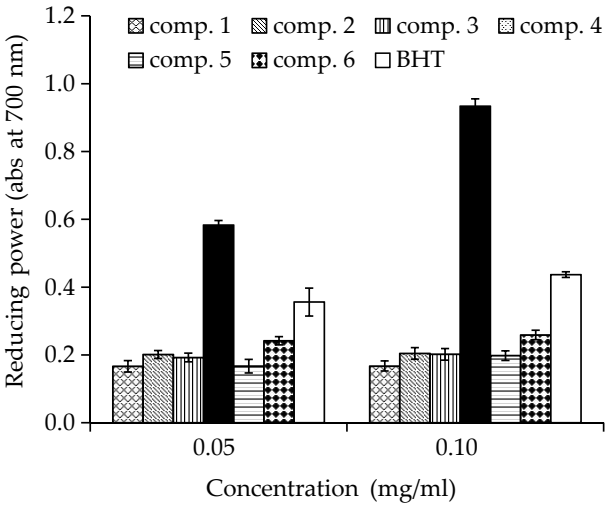

Figure 2. Reducing power of compounds isolated from nutmeg

Antioxidant activity assayed by ABTS$^{\bullet+}$ method

The ABTS$^{\bullet+}$ scavenging assay employs the absorbance at a wavelength (734 nm) remote from the visible region and requires a short reaction time. This method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts, and can be an index reflecting the antioxidant activity of the test samples (Re *et al.* 1999). Hence, the isolated compounds from nutmeg were measured to compare their capacities to scavenge ABTS$^{\bullet+}$ with that of BHT. As shown in Figure 3, compound 4 had a stronger antioxidant activity than was that of BHT. As its concentration increased from 0.02 mg/ml to 0.04 mg/ml, the scavenging effect increased from 51.2% to 75.9%. The activities of scavenging ABTS$^{\bullet+}$ were moderate for compounds 2 and 3, low for compounds 1 and 5–6, while in the case of compound 4 the activity was high. The free-radical scavenging abilities of the test samples decreased in the following order: compound 4 > BHT > compound 2 > compound 3 > compounds 6–5 > compound 1 (Figure 3).

Antioxidant activity assayed by DPPH$^{\bullet}$ scavenging method

The DPPH$^{\bullet}$ is characterised as a stable radical owing to the delocalisation of the spare electron, with maximum absorption at about 517 nm. The DPPH$^{\bullet}$ scavenging activity has been widely used to evaluate the antiradical activities of various samples (Molyneux 2004). As seen in Figure 4, among the test

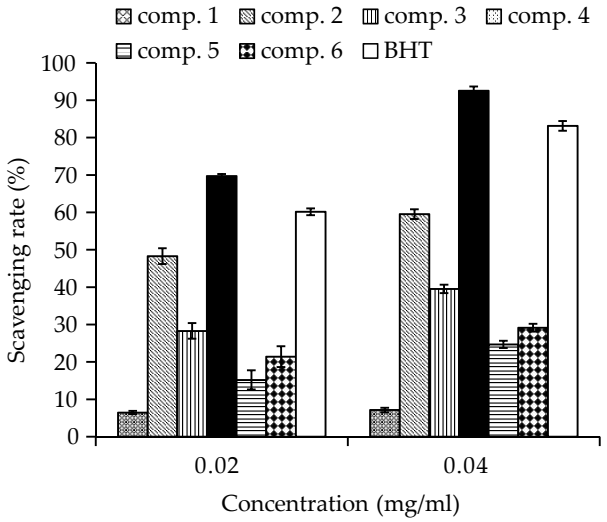

Figure 3. ABTS$^{\bullet+}$ scavenging activity (%) of compounds isolated from nutmeg

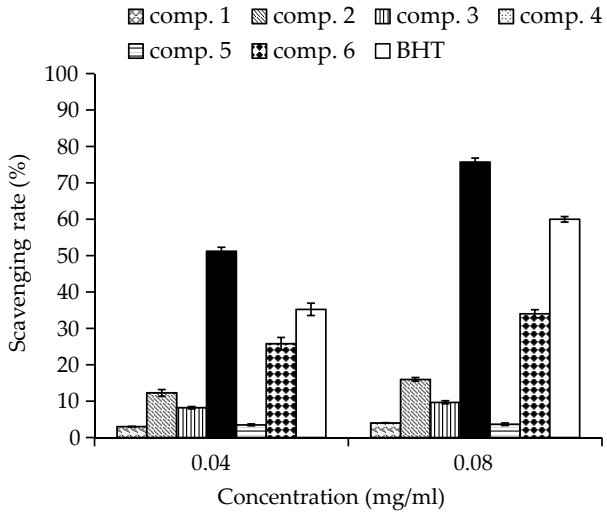

Figure 4. DPPH$^{\bullet}$ scavenging activity (%) of compounds isolated from nutmeg

samples, compound 4 showed a notably high radical scavenging activity. The other compounds showed obviously lower scavenging activities than BHT.

With regard to the scavenging activity toward radicals, compound 4 showed scavenging activities against both ABTS$^{\bullet+}$ and DPPH$^{\bullet}$. As for compounds 2 and 3, they both had the ability to scavenge ABTS$^{\bullet+}$ (Figure 3), but showed little scavenging activities against DPPH$^{\bullet}$ (Figure 4). One of the probable causes for this difference is that the rate of radicals reaction varies widely with different substrates (Brand-Williams *et al*. 1995; Bondet *et al*. 1997).

Among all the isolated compounds, only compound 4, malabaricone C, showed a strong antioxidant activity in each of the methods followed. Compound 4 possesses *o*-dihydroxyl groups in the benzene ring, which explains its high antioxidant activity. Previous studies reported other non-volatile constituents of nutmeg and mace (Woo *et al*. 1987; Hada *et al*. 1988; Leela 2008). Most of these constituents are phenolic compounds with more methoxyl groups and fewer hydroxyl groups, which have a lower antioxidant activity than the compounds possessing *ortho*-dihydroxyl groups. Our study showed that malabaricone C is one of the most important compounds contributing to the strong antioxidant activity of nutmeg.

CONCLUSION

Malabaricone B has been identified in nutmeg for the first time. Malabaricone C exhibited a stronger antioxidant activity than the commonly used synthetic antioxidant BHT in the oil stability index (OSI, Table 1), reducing power (Figure 2), ABTS$^{\bullet+}$ scavenging (Figure 3), and DPPH$^{\bullet}$ scavenging (Figure 4) methods. This compound may be used as a novel natural antioxidant in the food.

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