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Evaluation of antioxidant activity of parsley (*Petroselinum crispum*) essential oil and identification of its antioxidant constituents

Hui Zhang [a,b,*], Feng Chen [b], Xi Wang [c], Hui-Yuan Yao [a]

[a] *School of Food Science and Technology, Southern Yangtze University, 170 Huihe Road, Wuxi 214036, Jiangsu Province, PR China*
[b] *Department of Food Science and Human Nutrition, Clemson University, Clemson, SC 29634, USA*
[c] *Department of Genetics, Biochemistry, and Life Science Studies, Clemson University, Clemson, SC 29634, USA*

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Abstract

Antioxidant capacities of the essential oil extracted from parsley (*Petroselinum crispum*) were evaluated by three different in vitro assays: β-carotene bleaching assay, DPPH· free radical scavenging assay and Fe^{2+}-metal chelating assay. Results showed that the parsley oil (PO) possessed a certain degree of antioxidant activities in terms of β-carotene bleaching capacity and free radical scavenging activity, but its metal chelating capacity was negligible. The antioxidant EC_{50} values of the β-carotene bleaching assay and DPPH· free radical scavenging assay of the crude PO dissolved in methanol were measured in about 5.12 and 80.21 mg/mL, respectively. However, these values were much weaker than those of BHT in 0.01 and 0.58 mg/mL, and of α-tocopherol in 0.01 and 0.10 mg/mL. Isolation and identification of the inherent antioxidants in PO involved using various chromatographic techniques including silica gel open column chromatography, normal phase-HPLC and GC–MS. Myristicin in PO was found as a dominant compound (32.75%) that exhibited a moderate antioxidant activity. Apiol was the second dominant compound (17.54%), but it might be the major contributor to the antioxidant activity of PO. These results suggest that the PO and its two major components can be potential alternative natural antioxidants.
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Keywords: Parsley; *Petroselinum crispum*; Essential oil; Antioxidant activity; Apiol; Myristicin

1. Introduction

Free radicals cause the oxidation of biomolecules (e.g., protein, amino acids, lipid and DNA) which leads to cell injury and death (Freidovich, 1999; Ignarro, Cirino, Casini, & Napoli, 1999; Lander, 1997; McCord, 2000; Zheng & Storz, 2000). For example, reactive oxygen species (ROS) markedly alter the physical, chemical, and immunological properties of superoxide dismutase (SOD), which further exacerbates oxidative damage in cells. This has raised the possibility that antioxidants could act as prophylactic agents. It has long been recognized that naturally occurring substances in higher plants have antioxidant activities.

Antioxidants are also important to the food industry. Manufacturers have strived to produce high quality food with superior texture, color, flavor and nutritional values in the shelf life period. However, many foods are subject to many factors that lead to the quality deterioration. Among these undesirable factors, lipid autooxidation is one of the most concerned. The need of protecting food against oxidative degradation has prompted the wide usage of food additives. Although there are some synthetic antioxidant compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), these compounds are associated with some side effects (Ito, Fukushima, Hagiwara, Shibata, & Ogiso, 1983). Many studies have been shown that the presence of natural antioxidants from various aromatic and medicinal plants is closely related to the reduction of chronic diseases such as DNA damage, mutagenesis, and carcinogenesis (Briskin, 2000; Covacci et al., 2001; Craig, 1999; Reddy, Odhav, & Bhoola,

* Corresponding author. Address: School of Food Science and Technology, Southern Yangtze University, 170 Huihe Road, Wuxi 214036, Jiangsu Province, PR China. Tel.: +86 510 5869382; fax: +86 510 5874723.
E-mail address: hui1003@hotmail.com (H. Zhang).

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2003; Wargovich, 2001; Wargovich, Woods, Hollis, & Zander, 2001; Zhu, Hackman, Ensunsa, Holt, & Keen, 2002). Therefore, there has been a growing interest in research concerning alternative antioxidant active compounds, including plant extracts and essential oils that are relatively less damaging to the mammalian health and environment.

Essential oils are known to possess multifunctional properties other than their classical roles as natural food additives and/or fragrances. Besides the antibacterial, antifungal, and anti-inflammatory activities (Angioni et al., 2004; Bendimerad et al., 2005; Cardenas-Ortega, Zavala-Sanchez, Aguirre-Rivera, Perez-Gonzalez, & Perez-Gutierrez, 2005; Fernandez-Ocana et al., 2004; Gulluce et al., 2003; Hammer, Carson, & Riley, 1999; Koutsoudaki, Krsek, & Rodger, 2005; Meepagala, Sturtz, & Wedge, 2002; Singh, Maurya, Catalan, & de Lampasona, 2004; Yu et al., 2003), many essential oils also have been confirmed to possess the antioxidant activity (Kim et al., 2004; Miguel et al., 2004; Ramirez, Senorans, Ibanez, & Reglero, 2004; Ruberto & Baratta, 2000; Sokmen et al., 2005; Takahashi, Inaba, Kuwahara, & Kuki, 2003). Parsley (*Petroselinum crispum*) is native to Europe and Western Asia and cultivated in the United States as an annual for its aromatic and attractive leaves (Simon & Quinn, 1988). The three main types of parsley are the plain leaf type (ssp *neapolitanum*, Danert) and the curly leaf type (ssp *crispum*), which are cultivated for their foliage, and the turnip-rooted or 'Hamburg' type (ssp. *tuberosum*), primarily grown for its roots (Petropoulos, Daferera, Akoumianakis, Passam, & Polissiou, 2004). Fresh, dried, and dehydrated leaves are used as a condiment, garnish, and flavoring ingredient. An essential oil can be extracted from the leaves and seeds; it is used as a flavoring agent or fragrance in perfumes, soaps, and creams. The commercial essential oil of parsley is largely derived from the seed or the herb harvested at seed formation, prior to ripening (Petropoulos et al., 2004). Simon, Chadwick, and Craker (1984) studied the essential oils of 104 accessions from the USDA Plant Introduction Station and identified 1,3,8-*p*-menthatriene, myristicin, β-phellandrene and myrcene as the principal components. Zheng, Kenney, Zhang, and Lam (1992) found that as a major volatile aroma constituent of parsley essential oil, myristicin may be an effective cancer chemopreventive agent. Gazzani (1994) found that parsley showed weak antioxidant activity in groundnut oil under various heating conditions. Fejes et al. (1998) and Wong and Kitts (2006) investigated the in vitro antioxidant effect of various extracts prepared from different vegetative organs of parsley and observed that the essential oil plays a significant role in the scavenging effect. Therefore, the aim of this study is to investigate the antioxidant capacity of the parsley oil (PO) and identify its antioxidant components.

In this study, the antioxidant capacity of PO was investigated using three complimentary in vitro assays: β-carotene bleaching assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and the Fe^{2+}-metal chelating assay. The β-carotene bleaching and the radical scavenging activity of the crude PO was compared with that of the commercial standard antioxidants, butylated hydroxytoluene (BHT) and R-tocopherol, through the β-carotene bleaching assay and the DPPH$^{\cdot}$ free radical scavenging assay. Meanwhile, the metal chelating capacity between PO and ethylenediaminetetraacetic acid (EDTA) was compared. Moreover, some PO components showing antioxidant activity were further separated from the crude PO by silica gel column chromatography and Spherisorb silica HPLC and identified by GC–MS.

2. Materials and methods

2.1. Materials and chemicals

The crude PO, dry silica gel (70–230 mesh, 60 Å), 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$), β-carotene, linoleic acid, α-tocopherol, and BHT were purchased from Sigma Chemical Co. (St. Louis, MO). Ferrous chloride and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine) were purchased from Fluka Chemical Co (Milwakee, WI). EDTA and all HPLC analytical grade solvents were from Fisher Scientific (Suwanee, GA).

2.2. Gas chromatography–mass spectrometry

The Shimadzu's GC–MS system consisting of a GC-17A with QP5050 Mass Spectrometer (Kyoto, Japan) was equipped with a DB-5 capillary column (60 m × 0.25 mm, thickness 0.25 μm; J&W Scientific, Folsom, CA, USA) for all chemical quantitative and qualitative analysis in this experiment. The oven temperature was programmed from 40 to 300 °C at the ramp of 10 °C/min and held at 300 °C for 10 min. The injector and ion source temperatures were set at 250 °C. The detector voltage was 70 eV and the MS spectra were scanned in the mass range of m/z 43–350. Helium was used as the carrier gas and the column flow rate was 1.1 mL/min. The sample (2 μL) was injected into the capillary column with a split ratio of 2:1. Identification of compounds was based on comparison of their mass spectra and retention indices (RIs) with those recorded in the Wiley and NIST mass spectral databases, the previously published RIs, and the authentic standards. RIs were calculated using a series of n-alkanes (C_8–C_{20}). Quantitative analysis of each essential oil component (expressed in percentages) was carried out by peak area normalization measurement.

2.3. Antioxidative capacity

The antioxidant capacities of PO and its separated antioxidative components were determined by three methods: β-carotene bleaching assay, DPPH$^{\cdot}$ free radical scavenging assay and metal chelating assay. Two standard antioxidants (i.e., BHT and α-tocopherol) were used as the control for DPPH$^{\cdot}$ free radical scavenging assay, while EDTA was used as the control for metal chelating assay.

2.3.1. β-Carotene bleaching assay

This experiment was carried out by measuring the coupled autoxidation of β-carotene and linoleic acid (Sarkar, Bishayee, & Chatterjee, 1995). β-Carotene (5 mg) was dissolved in 50 mL of chloroform, and 3 mL was added to 40 mg of linoleic acid and 400 mg of Tween 20. Chloroform was gently removed under a stream of nitrogen gas. Distilled water (100 mL) was added and mixed well with the remaining emulsion. Aliquots (1.5 mL) of the β-carotene/linoleic acid emulsion were mixed with 20 μL of the PO or standards samples dissolved in methanol, and incubated in a water bath at 50 °C for 60 min. Oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Control samples contained 20 μL of solvent in place of the PO sample. The antioxidant activity is expressed as percent inhibition relative to the control after a 60 min incubation using the following equation:

$$\mathrm{AA}\ (\%) = 100(\mathrm{DR_C} - \mathrm{DR_S})/\mathrm{DR_C} \quad (1)$$

where AA is the antioxidant activity, DR_C is the degradation rate of the control: $\ln(a/b)/60$, DR_S is the degradation rate of the sample: $\ln(a/b)/60$, a is the initial absorbance at time 0, and b is the absorbance at 60 min.

2.3.2. DPPH· free radical scavenging assay

To determine the free radical scavenging activity of the crude PO and its separated fractions, DPPH· free radical scavenging assay reported by Yamaguchi, Takamura, Matoba, and Terao (1998) with minor modification was used. The reaction mixture containing 0.1 mL of sample dissolved in methanol, 0.3 mL of methanol, and 0.4 mL of 0.3 mM DPPH· reagent dissolved in methanol was vigorously shaken and incubated in the darkness at room temperature for 30 min. After incubation, the absorbance of the reaction mixture was measured spectrophotometrically at 517 nm. The scavenging effect of DPPH· free radical was calculated by using the following equation:

$$\text{Scavenging effect}\ (\%) = \left(1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100 \quad (2)$$

2.3.3. Metal chelating assay

The metal chelating effect of the crude PO and EDTA were determined by the ferrous ion chelating assay modified from the method of Dinis, Madeira, and Almeida (1994). A reaction solution composed of 600 μL of the sample dissolved in methanol and 40 μL of 2 mM $FeCl_2$ was activated by the addition of 80 μL of 5 mM ferrozine. After vortex, the reaction mixture was incubated at room temperature for 10 min, and its chelating activity was spectrophotometrically measured at 562 nm. The metal chelating effect was calculated by using the following equation:

$$\text{Chelating effect}\ (\%) = \left(1 - \frac{\text{absorbance of sample at 562 nm}}{\text{absorbance of control at 562 nm}}\right) \times 100 \quad (3)$$

EC_{50} value used to evaluate antioxidant capacities of the crude PO and standards were the effective concentration at which the AA, scavenging effect or chelating effect were 50%.

2.4. Fractionation and identification of antioxidants from PO

2.4.1. Silica gel column chromatography

A glass column (60 × 2.5 cm) packed with the silica gel and maintained at 25 °C with a jacket was equilibrated with ethyl acetate. Two milliliters of the crude PO dissolved in ethyl acetate was loaded and eluted by multi-step solvent gradients as follows: ethyl acetate, ethyl acetate/methanol (1:1, v/v), and methanol. The flow rate was 4 mL/min and each collected fraction was 4 mL. After the open-column separation by silica gel column chromatography, those PO fractions bearing free radical scavenging activity determined by DPPH· assay were pooled for further analyses.

2.4.2. HPLC Separation

A Spherisorb silica column (250 × 4.6 mm, 5 μm; Waters, MA, USA) was connected with a Shimadzu LC-10AT HPLC system (Kyoto, Japan) and equilibrated with hexane/DCM (1:1, v/v). Twenty microliters of the pooled samples were injected into the HPLC column and eluted at a flow rate of 1 mL/min by hexane/DCM (1:1, v/v). All peaks of the eluant were collected after passing through a Shimadzu SPD-M10V photodiode array detector (Kyoto, Japan). The scan range of absorbance for the eluant was from 200 to 300 nm.

2.5. Statistical analysis

Mean values of listed data were average of triplicates. The data of the antioxidant activities of the crude PO and its components were subjected to the analysis of variance (ANOVA).

3. Results and discussion

3.1. PO composition

As shown in Table 1, the parsley essential oil in current analysis is characterized by the dominant presence of five substance: myristicin (32.75%), apiol (17.54%), α-pinene (16.64%), β-pinene (11.54%) and 1-allyl-2,3,4,5-tetramethoxy-benzene (10.00%). Although, in general, the major constituent in parsley essential oil was 1,3,8-*p*-menthatriene, followed by β-phellandrene, myristicin, apiol and myrcene (Simon et al., 1984), we did not find 1,3,8-*p*-menthatriene and myrcene in our analysis. This may due to the plant genetic base and development and environmental conditions.

3.2. Determination of antioxidant activity of the crude PO

The antioxidant capacity of the crude PO was initially determined by the β-carotene bleaching assay. The EC_{50}

Table 1
Chemical composition of crude PO

Retention time (min)	Compound[a]	Composition (%)	RI[b]
8.60	Unknown	0.22	909
9.16	α-Pinene	16.64	943
9.82	Sabinene	0.49	981
9.97	β-Pinene	11.54	989
10.68	*p*-Cymene	0.26	1032
10.78	Limonene	0.64	1038
10.84	β-Phellandrene	4.24	1042
11.23	γ-Terpinene	0.30	1066
11.78	*p*-Cymenene	0.31	1097
13.55	Unknown	0.17	1212
17.98	Myristicin	32.75	1529
18.12	Elemicin	4.13	1550
18.63	1-Allyl-2,3,4,5-tetramethoxy-benzene	10.00	1591
19.13	Carotol	0.77	1615
19.78	Apiol	17.54	1690

[a] The components of PO were identified by their mass spectra and retention indices (RIs) with that of the Wiley and NIST mass spectral databases and the previously published RIs.
[b] RI were calculated using a series of *n*-alkanes (C_8–C_{20}).

value of the crude PO dissolved in methanol was about 5.12 mg/mL, though this antioxidant activity was much weaker than that of 0.01 mg/mL of BHT and α-tocopherol (Table 2). β-Carotene shows strong biological activity and is physiologically an important compound (Sarkar et al., 1995; Ziegler et al., 1996), if β-carotene is decomposed before its intake, its biological functions in the body would not be observed. However, its 11 pairs of double bonds are extremely sensitive to free-radical mediated oxidation, and it is discolored easily with oxidation of linoleic acid (Unten, Koketsu, & Kim, 1997). In the present study, we found that the crude PO has a relatively strong effect against the discoloration of β-carotene with linoleic acid.

Next, DPPH· free radical scavenging activity of crude PO was invested. The model system of scavenging DPPH· free radicals is a simple method to evaluate the antioxidative activity of antioxidants. It is accepted that the DPPH· free radical scavenging by antioxidants is due to their hydrogen-donating ability (Chen & Ho, 1995). PO exhibited the EC_{50} of DPPH· free radical scavenging activity at the concentration of 80.21 mg/mL, This value is much weaker than those of BHT and α-tocopherol at 0.58 and 0.10 mg/mL, respectively (Table 2).

In the ferrous ion metal-chelating test, the EC_{50} value of EDTA was 0.02 mg/mL, the inhibitive capacity of the PO against the metal chelating was not detected in all prepared concentrations.

So, the results shown in Table 2 indicate that the crude PO is moderately competitive as a β-carotene bleaching and free radical scavenging agent to the well-known synthetic antioxidants such as BHT and α-tocopherol, but it may be not a material to prevent free radical reactions initiated by metal ions.

Table 2
Antioxidant capacities of the crude PO and three standard antioxidants

	Antioxidant capacity (EC_{50}, mg/mL)		
	Scavenging activity	Metal chelating activity	AA
Crude parsley oil	80.21 ± 3.41	NA[c]	5.12 ± 0.26
BHT[a]	0.58 ± 0.02		0.01 ± 0.00
α-Tocopherol[a]	0.10 ± 0.00		0.01 ± 0.00
EDTA[b]		0.02 ± 0.00	

[a] BHT and α-tocopherol were used as standards for DPPH· free radical scavenging and β-carotene bleaching assay.
[b] EDTA was a standard for metal chelating assay.
[c] Not available.

3.3. Separation and identification of antioxidants from the crude PO

Since the crude PO exhibited an antioxidant capacity in the DPPH· and the β-carotene bleaching test, the inherent antioxidants in the crude PO were then separated sequentially with silica gel open-column chromatography, silica gel-HPLC, and finally identified by GC–MS.

When the crude PO was separated by the silica gel open column chromatography using a stepwise solvent elution method with ethyl acetate, ethyl acetate/methanol (1:1, v/v) and methanol, a total of ninety fractions were collected. As shown in Fig. 1, there was only one antioxidant peak named PO-A with 51% scavenging effect from the DPPH· included the fractions 29 and 30 separated by a solvent mixture of ethyl acetate/methanol (1:1, v/v). For further separation, the 29 and 30 fractions with antioxidant activity were collected, removed solvent under a stream of nitrogen gas and redissolved in 1 mL of hexane. Then the PO-A redissolved in hexane was pooled and separated by the Spherisorb silica HPLC column. As shown in Fig. 2, there were three peaks named

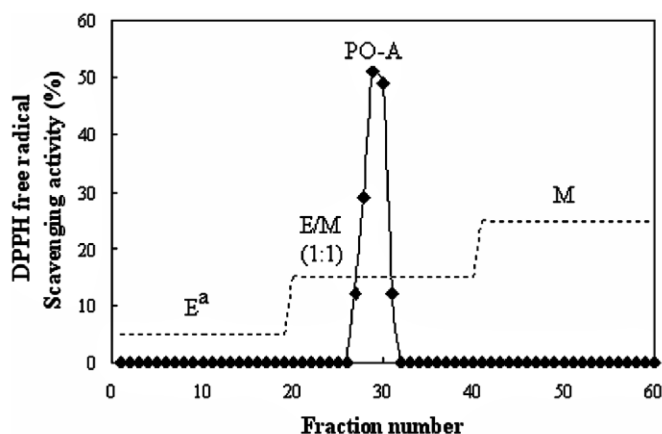

Fig. 1. Free radical scavenging activity of the fractions of PO separated by silica gel open column chromatography. Antioxidant activities of all collected fractions were determined by the DPPH· assay ([a]E, and M represent ethyl acetate and methanol, respectively).

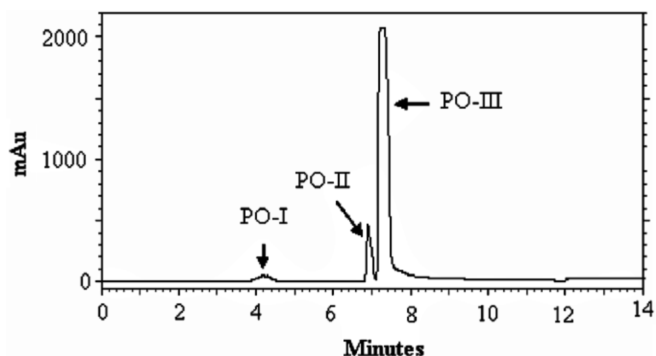

Fig. 2. Chromatograms of PO-A separated by Spherisorb silica-HPLC. The method was described in Section 2, the wavelength of absorbance was 250 nm.

PO-I, PO-II and PO-III in the same HPLC profile. These three peaks were collected for 15 times and concentrated under a stream of nitrogen gas to about 1 mL to measure the antioxidant activities of by the DPPH· assay. Peaks PO-II and PO-III have been found with 10.1% and 56.3% of DPPH· free radical scavenging activity in the DPPH· test. Thus, both the fractions of PO-II and PO-III were selected for further chemical separation and identification by GC–MS.

3.4. Identification of antioxidants in PO-II and PO-III separated by Spherisorb silica-HPLC

Further separation and identification of compounds in the two fractions revealed that the major compounds in PO-II and PO-III are myristicin and apiol, respectively (Fig. 3), which are the two dominant compounds in PO.

Myristicin is a kind of phenolic compound. A lot of phenolic compounds exhibit a wide range of biological effects as a consequence of their antioxidant properties. Several types of phenolic compounds (phenolic acids, hydrolysable tannins, and flavonoids) show anticarcinogenic and antimutagenic effects (Urqulaga & Leighton, 2000). Myristicin also was reported with anticarcinogenic effect, and was an effective cancer chemopreventive agent (Zheng et al., 1992). In our study, we found it had a moderate antioxidant activity with 12.01 mg/ml of the EC_{50} in the DPPH· free radical scavenging assay.

The DPPH· free radical scavenging activity of PO-III is more than 5-fold as that of PO-II, indicated that the antioxidant activity of apiol is much stronger than myristicin. Apiol has a very similar structure with myristicin, and has one more methoxy group on the symmetrical position of another methoxy group. As well known, the methoxy group is also a strong electron-donating group, which

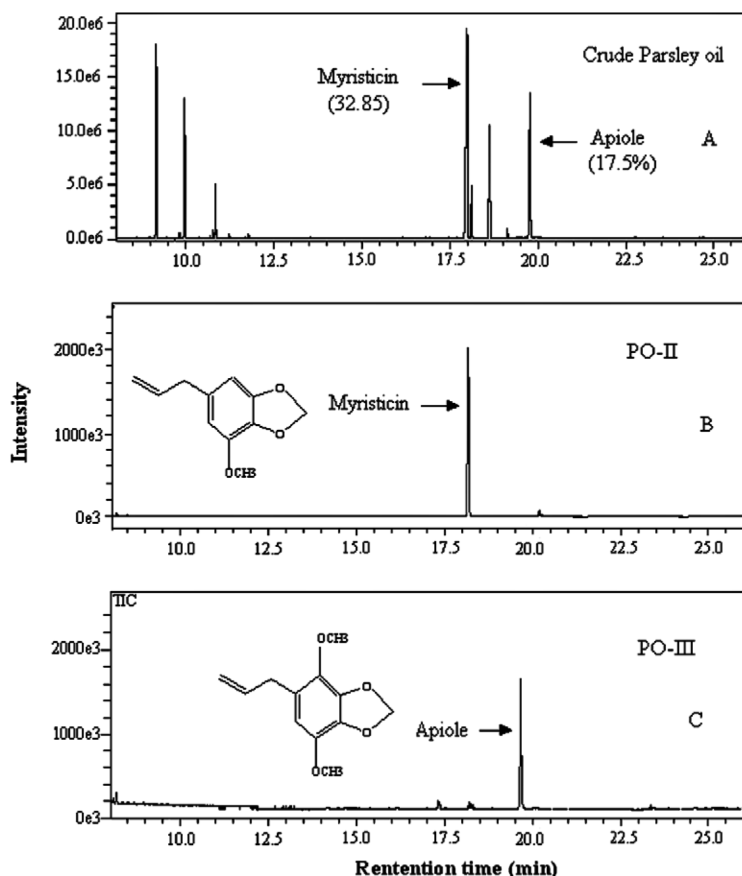

Fig. 3. Gas chromatographic profiles of the crude PO (A), PO-II (B) and PO-III (C), and identification of their major constituents. A DB-5 capillary column was installed in a Shimadzu GC-17A instrument that was also connected to a QP 5050 MS detector. The GC oven temperature was programmed from 40 to 300 °C at the rate of 10 °C/min and held at 300 °C for 10 min. The injector and ion source temperatures were 250 °C.

increases the stability of the benzene ring resulting in increase of radical scavenging activity. Benabadji, Wen, Zheng, Dong, and Yuan (2004) found that substitution on indole benzene ring with methoxy group had greatly enhance the radical scavenging activity of the unsubstitute compounds. The addition of a methoxy group also confers antioxidant activity to the chalcones (Miranda et al., 2000). So, as the second dominant compound, apiol is the major contributor to the antioxidant activity of PO. Myristicin, which is the first dominant compound, also plays an important role in the antioxidant activity of PO. However, due to the lack of enough standard apiol, we could not determine the synergistic effect between the myristicin and apiol, and other chemicals' effect on apiol.

In summary, the demonstration of PO and two of its inherent bioactive phenolic compounds having antioxidant activity was the most intriguing result of our study. To our knowledge, such results have never been reported. Moreover, anticancer functionalities of the phenolic compounds are underway in our lab.

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