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Chemical composition and antioxidant activity of volatiles from *Patrinia Villosa* Juss obtained by optimized supercritical fluid extraction

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ABSTRACT

Supercritical CO_2 fluid extraction (SFE-CO_2) was used to extract volatiles from *Patrinia Villosa* Juss. An orthogonal test L_9 $(3)^4$ including pressure, temperature, dynamic extraction time and modifier was performed to get the optimal conditions. Extract 1 was obtained by the optimal extraction condition 1: pressure = 35 MPa, T = 45 °C, dynamic extraction time = 2.0 h and $V_{\text{modifier (MeOH)}}$ = 0% as guided by the index 1: the free radical scavenging activities *in vitro* against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt (ABTS). Extract 2 obtained by the optimal extraction condition 2: pressure = 25 MPa, T = 55 °C, dynamic time = 2.5 h and $V_{\text{modifier (MeOH)}}$ = 20% was guided by the index 2: the yield of the volatiles. The results showed that extract 1 possessed stronger antioxidant activity (EC_{50} = 32.01 μg/ml to DPPH and 50.90 μg/ml to $ABTS^+$) than the extract 2 (EC_{50} = 95.62 μg/ml to DPPH and 99.78 μg/ml to $ABTS^+$). Subsequently, the chemical compositions of the two extracts were identified by gas chromatography–mass spectrometry. Forty-six compounds were identified from extract 1, and the total volatile consisted of hydrocarbon (49.65%), aldehyde (16.66%), fatty acid (22.38%), terpene (9.04%) and little alcoholic. From extract 2, 32 compounds were identified, in which hydrocarbon, aldehyde, fatty acid and terpene possessed 58.21%, 5.97%, 13.19% and 21.79%, respectively. This is the first report of using SFE to extract the volatiles from *P. Villosa* Juss (a wild vegetable and medicine plant) and first time to systematically evaluate the volatiles' antioxidant activity and chemical composition.

1. Introduction

The genus *Patrinia*, with about 20 species, which belongs to family Valerianaceae, is an herbaceous perennial plant natively grown in central to east of Asia and northeast of North America. Young leaves and flower buds of *Patrinia* species have been cooked and used as wild vegetables in some areas of China. The whole plant of *Patrinia* can be applied to medicine as anti-virus and anti-bacterial agent [1–3], especially two species, *Patrinia Scabiosaefolia* Fisch. and *Patrinia Villosa* Juss (*P. Villosa* Juss).

As for the chemical constituents of this genus, *P. scabiosaefolia* Fisch. [4], *Patrinia scabra* [5] and *Patrinia gibbosa* [6] have been more thoroughly investigated than *P. Villosa* Juss. Some iridoids, flavonoids and saponins in *P. Villosa* Juss have been studied [7–19] most of them since 2005 [9–19]. However, to the best of our knowledge, only two superficial studies of volatiles from *P. Villosa* Juss have been reported, which concerned the identification of five compounds in *P. Villosa* Juss [20,21].

Supercritical CO_2 fluid extraction (SFE-CO_2), using CO_2 instead of organic solvent and possessing unusual properties including high compressibility, liquid-like density, high diffusivity, low viscosity and low surface tension, can be considered one of the most potentially useful new methods of sample preparation in pharmaceutical and food processing industry [22–24]. This advanced extraction method has been successfully adopted to extract aurentiamide acetate from *P. Villosa* Juss in our previous study [10]. Many reports demonstrated that SFE-CO_2 was superior to some conventional methods, e.g. hydrodistillation, steam distillation and solvent extraction for the isolation of volatile compounds from medicinal plants [25–27]. The integration of SFE with gas chromatography–mass spectrometry (GC/MS) permits the rapid analysis of volatiles, which has been adopted in the study of chemical composition of herb medicines [28].

Many diseases are associated with free radicals because oxidative damage to DNA, proteins and other macromolecules accumulates with age and has been postulated to constitute a major type of endogenous damage leading to aging [29]. Although almost

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all organisms are equipped with antioxidant defense and repair systems that have involved protecting them against oxidative damage, these systems are often inadequate to completely prevent the damage [30]. However, antioxidant supplements, or natural products containing antioxidants, may be used to help reduce oxidative damage to human body. Many papers have been reported to find safe and potent natural antioxidants from various plant sources. As harmless sources of antioxidants, wild herbs, spices, fruits, nuts and leafy vegetables have been investigated [31–34]. Some volatiles or essential oils have been found to exhibit strong antioxidant activity [35–38].

The aim of the present paper, therefore, was to choose the optimal SFE conditions using an orthogonal test design to obtain the extracts, which possessed strong antioxidant activity. Then, the chemical compositions of the extracts obtained by the optimal SFE conditions were determined by GC/MS. The results as guided by the antioxidant activity were compared with those obtained under the selected condition as guided by the yield of the volatile. Therefore, scavenging effects, yield of the extract and chemical composition were obviously different according to different evaluation standards in SFE. To our best knowledge, this is the first report of using SFE to extract the volatiles from *P. Villosa* Juss, and first time to systematically evaluate the antioxidant activity and chemical composition of the extract.

2. Experimental

2.1. Chemicals and materials

The *P. Villosa* Juss was purchased from a local drug store and identified by Dr. Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China). To avoid degradation, the air-dried plant material was ground just before extraction.

Carbon dioxide (99.95%) was obtained from Beijing Analytical Instrument Factory. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt (ABTS), nonacosane, *n*-hexadecanoic acid and ascorbic acid were purchased from Sigma Chemical Co., USA. Other chemicals used were all analytical grade and purchased from WuLian Chemical Factory, Shanghai, China.

2.2. Supercritical fluid apparatus and extraction

A Suprex HA111-05-20 system (Hua An SFE Company Ltd., Nan Tong City, Jiang Su Province, China) in the SFE mode was used for optimization the extraction conditions. In this study, extractions were accomplished with 1000-ml volume extraction vessel. Nine extractions were carried out at 45, 55 and 65 °C, pressure of 15, 25 and 35 MPa, and dynamic extraction time of 1.5, 2.0 and 2.5 h. Two different concentrations of methanol (10% and 20%) were used as modifier. Table 1 shows the SFE experimental conditions for the extraction. The extract was trapped into a collection vessel by a Duraflow manual variable restrictor. In each test, exactly 200 g of the powder plant material was weighed and filled into the extraction vessel. The plant was then extracted with supercritical carbon dioxide under the nine conditions described in Table 1 and the extracted volatile was collected for further antioxidant activity assay. For all the modifier research, methanol was spiked directly into the extraction vessel with charged sample prior to extraction.

2.3. Antioxidant activity

2.3.1. DPPH stable free radical scavenging

The free radical scavenging activity of each SFE extract and ascorbic acid (control) in absolute ethanol were determined based on their ability to react with the stable DPPH free radical according to the literature [34]. In brief, a 750 μl of the SFE extract (from 10 to 150 μg/ml, dissolved in absolute ethanol) was added to 750 μl of DPPH in absolute ethanol (152 μM). After incubation at 37 °C for 20 min, the absorbance of each solution was determined at 520 nm using a Varian spectrophotometer. The concentration of sample required for 50% scavenging of the DPPH free radical (EC_{50}) was determined. The average 50% scavenging concentration was carried out in triplicate. The average 50% scavenging concentration was then calculated.

2.3.2. $ABTS^+$ radical cation scavenging

The $ABTS^+$ radical cation scavenging activity of each SFE extract and ascorbic acid (control) was determined according to the literature [34]. In brief, 5.0 ml, 7.0 mM ABTS was reacted with 88.0 μl, 140 mM potassium persulfate overnight in the dark to yield the $ABTS^+$ radical cation. Prior to use in the assay, the $ABTS^+$ radical cation was diluted with ethanol for an initial absorbance of about 0.700 (ratio of 1:88) at 734 nm, with 30 °C. Free radical scavenging activity was assessed by mixing 1.0 ml diluted $ABTS^+$ radical cation with 10 μl of test sample and monitoring the change in absorbance at 0, 0.5 and 1 min, and again 5 min intervals until a steady state was achieved. The antioxidant capacity of volatile was expressed as EC_{50}, the concentration necessary for 50% reduction of $ABTS^+$.

2.4. Optimization of the extraction condition and validation

The scavenging effects of each volatile and the extraction yield of volatiles were chosen as the two kinds of indexes to optimize the extraction conditions, and then be compared. Under these two kinds of optimal conditions, the validations of the antioxidant activity and the yield of the extract were carried out by triplicate experiments.

Table 1
$L_9\ (3)^4$ orthogonal test design

Run no.	Factors			
	A: pressure (MPa)	B: temperature (°C)	C: dynamic time (h)	D: modifier (MeOH %)
1	1 (15)	1 (45)	1 (1.5)	1 (0)
2	1 (15)	2 (55)	2 (2.0)	2 (10)
3	1 (15)	3 (65)	3 (2.5)	3 (20)
4	2 (25)	1 (45)	2 (2.0)	3 (20)
5	2 (25)	2 (55)	3 (2.5)	1 (0)
6	2 (25)	3 (65)	1 (1.5)	2 (10)
7	3 (35)	1 (45)	3 (2.5)	2 (10)
8	3 (35)	2 (55)	1 (1.5)	3 (20)
9	3 (35)	3 (65)	2 (2.0)	1 (0)

2.5. GC and GC/MS analysis

GC and GC–MS analysis of the volatile was performed on a Finnigan Voyager gas chromatograph coupled with a mass detector. The column used for volatile separation was a fused silica OB-5 column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). For MS detection, electron ionization mode with ionization energy of 70 eV was used. The oven temperature was programmed from 50 °C (isotherm for 2 min) to 250 °C at a rate of 10 °C/min. The injector temperature was set at 250 °C. Helium was used as carrier gas at a flow rate of 1.0 ml/min. The samples (1.0 μl) were injected using split mode (split ratio 1:30). The compounds were confirmed by computer matching of their mass spectral fragmentation patterns with those of compounds in NIST-MS Library.

2.6. Statistics and data processing

Results are expressed as mean ± standard deviation (S.D.). The statistical analysis was carried out using the Student's *t*-test for paired data.

3. Results and discussion

Steam distillation has traditionally been applied for volatiles recovery from plant materials. In our initial study, the hydrodistillation method was used to extract the volatile compounds from *P. Villosa* Juss. However, because one of the disadvantages of the hydrodistillation methods is that volatiles undergo chemical alteration and the heat-sensitive compounds can easily be destroyed, the quality of the volatile extracts was poor after distilled for 8 h. Thus, SFE-CO_2 method, having relatively low critical pressure and temperature, was considered to be adopted.

The first step in the SFE of volatiles is to optimize the operating conditions to obtain an efficient extraction. The fluid pressures, modifiers, dynamic extraction time and temperature are generally considered as the most important factors to affect the extraction process. In the present study, all selected factors were examined using an orthogonal L_9 $(3)^4$ test design.

Generally, the optimization of the extraction condition was performed as guided by the yield of the volatile or the chemical composition. In the present paper, the optimized extraction condition in SFE was obtained according to the antioxidant activity of each extract against the DPPH and ABTS free radical assay. The antioxidant capacity of volatiles was expressed as EC_{50} (concentration of antioxidant required to quench 50% of the stable free radical), which was used to acquire the optimized extraction condition. The results presented in Table 2 indicated that the strongest antioxidant activity of EC_{50} values of the volatiles were 35.51 μg/ml to DPPH and 52.56 μg/ml to $ABTS^+$. As positive controls, the EC_{50} measured values against DPPH and $ABTS^+$ of ascorbic acid were 5.88 and 7.45 μg/ml, respectively, which indicated that the antioxidant activity of the volatiles in *P. Villosa* Juss were lower than that of ascorbic acid.

In our experiment, using two concentrations of methanol solution as modifier (D), the EC_{50} and extraction yield at different sets of pressure (A), temperature (B) and dynamic extraction time (C) were examined under design. The results of L_9 $(3)^4$ test shown in Table 2 revealed great difference between each set of SFE condition. The EC_{50} and yield data were analyzed and listed in Table 3. It was demonstrated that the influence of modifier to the antiox-

Table 2
L_9 $(3)^4$ test results

Test no.	A	B	C	D	Scavenging effects[a]	Scavenging effects[b]	Yield of the volatiles (%)[c]
1	A_1	B_1	C_1	D_1	41.52	52.56	0.33
2	A_1	B_2	C_2	D_2	88.65	89.25	1.06
3	A_1	B_3	C_3	D_3	116.47	122.64	1.60
4	A_2	B_1	C_2	D_3	88.63	72.36	1.79
5	A_2	B_2	C_3	D_1	47.68	55.64	1.65
6	A_2	B_3	C_1	D_2	76.23	99.21	1.30
7	A_3	B_1	C_3	D_2	68.57	62.69	1.69
8	A_3	B_2	C_1	D_3	94.65	95.92	2.01
9	A_3	B_3	C_2	D_1	35.51	57.45	1.00

[a] Antioxidant activity against 50% DPPH scavenging activity expressed as the average EC_{50} (μg/ml, $n = 3$).
[b] Antioxidant activity against 50% $ABTS^+$ scavenging activity expressed as the average EC_{50} (μg/ml, $n = 3$).
[c] Extraction yield of the volatiles (%) = the amount of volatiles/sample mass ($n = 3$).

Table 3
Analysis of L_9 $(3)^4$ test results

	Antioxidant activity (EC_{50}, μg/ml)[a]				Antioxidant activity (EC_{50}, μg/ml)[b]				Yield of volatiles (%)[c]			
	A	B	C	D	A	B	C	D	A	B	C	D
K_1[d]	246.64	198.72	212.40	124.71	264.45	187.61	247.69	165.65	2.99	3.81	3.64	2.98
K_2	212.54	230.98	212.79	233.45	227.21	240.81	219.06	251.15	4.74	4.72	3.85	4.05
K_3	198.73	228.21	232.72	299.75	216.06	279.30	240.37	290.92	4.79	3.90	4.94	5.40
k_1[e]	82.21	66.24	70.80	41.57	88.15	62.54	82.56	55.22	1.00	1.27	1.21	0.99
k_2	70.85	76.99	70.93	77.82	75.74	80.27	73.02	83.72	1.58	1.57	1.28	1.35
k_3	66.24	76.07	77.57	99.92	72.02	93.10	80.32	96.97	1.60	1.30	1.65	1.80
R[f]	15.97	9.83	6.77	58.35	16.13	30.56	9.54	41.75	0.60	0.30	0.44	0.81
Optimal level	A_3	B_1	C_1	D_1	A_3	B_1	C_2	D_1	A_2	B_2	C_3	D_3

[a] Antioxidant activity against 50% DPPH scavenging activity expressed as the average EC_{50} (μg/ml, $n = 3$).
[b] Antioxidant activity against 50% $ABTS^+$ scavenging activity expressed as the average EC_{50} (μg/ml, $n = 3$).
[c] Extraction yield of the volatiles (%) = the amount of volatile/sample mass ($n = 3$).
[d] $K_i^A = \Sigma EC_{50}$ or extraction yield of volatiles at A_i.
[e] $k_i^A = \frac{K_i^A}{3}$.
[f] $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

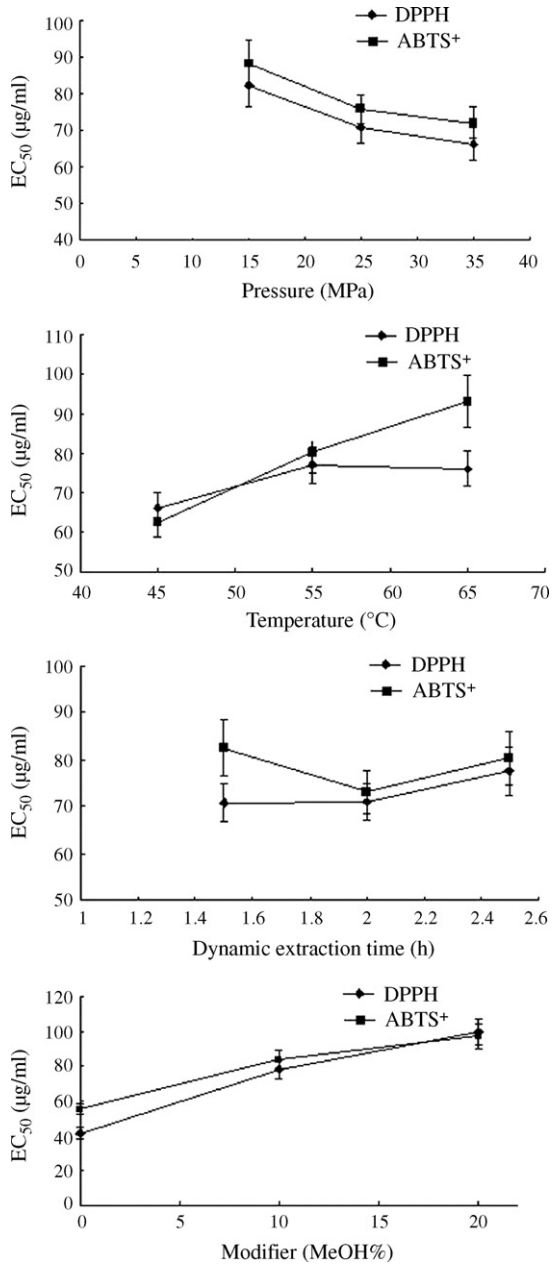

Fig. 1. The effect of pressure, temperature, dynamic time and modifier on the antioxidant activity of the volatiles. DPPH, 1,1-diphenyl-2-picrylhydrazyl; $ABTS^+$, 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt; EC_{50}, concentration of antioxidant required to quench 50% of the stable free radical ($n = 3$).

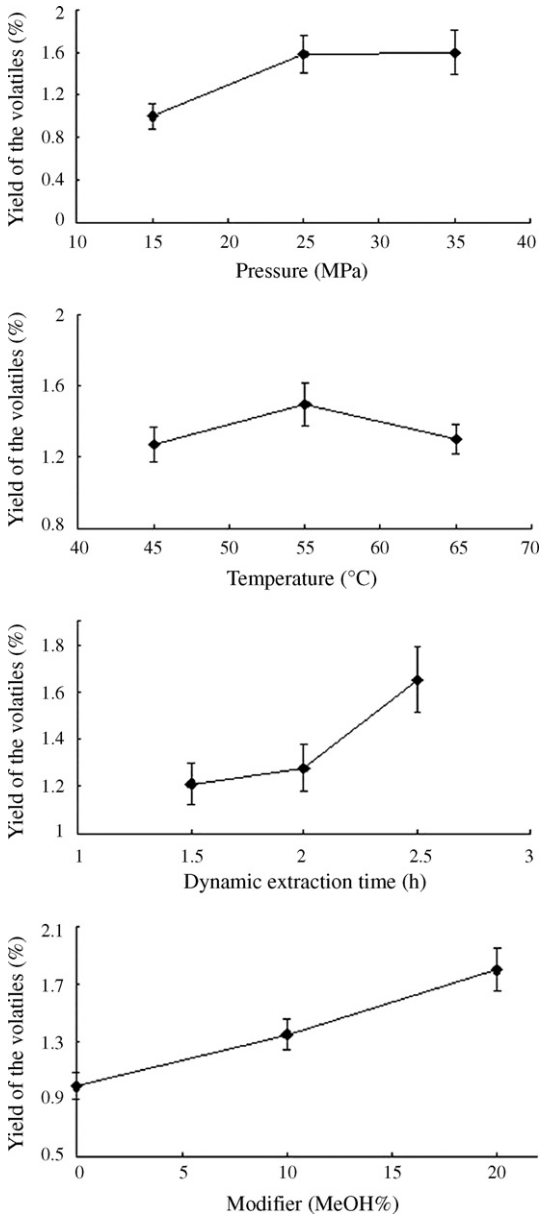

Fig. 2. The effect of pressure, temperature, dynamic extraction time and modifier on the extraction yield of the volatiles. Yield of the volatile (%) = (the amount of the volatile (g)/the sample mass (g)) × 100% ($n = 3$).

idant activity was most significant among these four parameters. The influence of the selected parameters was D>A>B>C to DPPH and D>B>A>C to $ABTS^+$. Lower EC_{50} could be obtained without modifier in SFE. High pressure, low temperature and a moderate dynamic extraction time are satisfactory, but low pressure and high temperature seem unfavorable for our aim. With regard to the yield of the volatiles, the influence of the selected parameters was D>A>C>B, and high concentration of modifier, long extraction time, moderate pressure and temperature were advantageous to obtain high yield. The influences of four parameters in three levels to EC_{50} values and the extraction yield of the volatiles are shown in Figs. 1 and 2, respectively.

The optimal SFE conditions were $A_3B_1C_1D_1$ and $A_3B_1C_2D_1$ according to the EC_{50} values against DPPH and $ABTS^+$, respectively. But there was no difference between C_1 and C_2 to the EC_{50} values against DPPH, therefore, the optimal SFE extraction condition was $A_3B_1C_2D_1$ (extraction condition 1) according to the scavenging activity. Meanwhile, another optimal SFE extraction condition was $A_2B_2C_3D_3$ (extraction condition 2) based on the yield of the volatile. Under the optimized extraction condition 1 of pressure = 35 MPa, $T = 45\,^\circ C$, dynamic extraction time = 2.0 h and CO_2 fluid modified with no methanol, the extract processed stronger antioxidant activity (32.01 μg/ml to DPPH and 50.90 μg/ml to $ABTS^+$) than the extract obtained under the optimized extraction condition 2 of pressure = 35 MPa, $T = 55\,^\circ C$, dynamic extraction time = 2.5 h and $V_{\text{modifier (MeOH)}} = 20\%$ (95.62 μg/ml to DPPH and 99.78 μg/ml to $ABTS^+$). On the contrary, the extraction yield obtained under extraction condition 1 (0.84%) was much lower than that obtained under extraction condition 2 (2.14%). The comparisons of antioxidant activity and extraction yield under different extraction conditions are shown in Fig. 3.

Table 4
Chemical constituents of volatiles extracted by SFE using GC/MS

Serial no.	RT (min)	KI[a]	Compound	Formula	Area (%)[b]	Area (%)[c]
1	2.50	1,420	Heptane	C_7H_{16}	1.02	0.04
2	3.05	33,650	(*S*)-5-Hydroxymethyl-2[5*H*]-furanone	$C_5H_6O_3$	0.09	–
3	3.33	3,430	1-Pentanol	$C_5H_{12}O$	0.09	–
4	3.57	33,660	2-Butenal	C_5H_8O	0.04	–
5	3.72	1,413	2-Methyl-1-pentanol	$C_6H_{14}O$	0.15	–
6	3.78	5,470	2,4-Dimethylhexane	C_8H_{18}	5.11	1.26
7	4.23	6,300	2,3-Dimethyl-1-butanol	$C_6H_{14}O$	0.15	–
8	4.70	6,540	5,9-Dodecadien-2-one	$C_{14}H_{24}O$	0.20	–
9	5.49	2,730	*n*-Heptaldehyde	$C_7H_{14}O$	0.25	–
10	6.45	1,456	Hept-*cis*-2-enal	$C_7H_{12}O$	1.28	0.35
11	7.08	31,870	2-*n*-Pentylfuran	$C_9H_{14}O$	0.14	0.06
12	7.26	698	*n*-Caprylaldehyde	$C_8H_{16}O$	0.42	0.12
13	7.88	13,590	3,5-Octadien-2-ol	$C_8H_{14}O$	0.18	0.03
14	8.20	737	(*E*)-2-Octen-1-al	$C_8H_{14}O$	0.54	0.02
15	8.40	819	Octyl alcohol	$C_8H_{18}O$	0.39	0.31
16	8.96	4,820	*n*-Nonaldehyde	$C_9H_{18}O$	2.63	1.86
17	9.84	690	(*E*)-2-Nonenal	$C_9H_{16}O$	0.21	–
18	9.98	5,580	*n*-Caprylic acid	$C_8H_{16}O_2$	0.38	0.59
19	10.54	1,463	*n*-Decaldehyde	$C_{10}H_{20}O$	0.36	–
20	11.18	1,756	(*Z*)-2-Decenal	$C_{10}H_{18}O$	0.56	–
21	11.39	1,756	(*E*)-trans-2-Decenal	$C_{10}H_{18}O$	6.44	2.34
22	11.85	31,387	(*E*,*E*)-(*E*,*E*)-2,4-Decadienal	$C_{10}H_{16}O$	0.38	0.02
23	12.17	31,390	(*E*,*E*)-2,4-Decadienal	$C_{10}H_{16}O$	0.63	0.09
24	12.61	23,720	Undec-2-enal	$C_{11}H_{20}O$	0.33	0.21
25	12.82	237,190	*trans*-2-Undecen-1-al	$C_{11}H_{20}O$	1.77	0.87
26	14.29	25,610	9-Oxononanoic acid	$C_9H_{16}O_3$	0.78	0.54
27	15.31	25,780	*n*-Dodecanoic acid	$C_{12}H_{24}O_2$	0.42	0.31
28	16.28	13,270	Tridecylic acid	$C_{13}H_{26}O_2$	0.61	0.04
29	17.61	6,977	Tetradecanoic acid	$C_{14}H_{28}O_2$	0.87	–
30	18.49	31,860	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	0.41	–
31	18.55	5,795	Hexahydrofarnesyl acetone	$C_{18}H_{36}O$	0.50	–
32	19.35	4,647	3,7,11-Trimethyl-hexa-hydro-farnesol	$C_{15}H_{32}O$	0.79	0.42
33	19.75	2,145	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	14.98	5.69
34	21.42	13,230	9-Octadecenoic acid	$C_{18}H_{34}O_2$	1.13	2.25
35	21.49	3,396	1-Heptadecene	$C_{17}H_{34}$	0.55	0.98
36	21.63	6,590	Octadecanoic acid	$C_{18}H_{36}O_2$	2.52	2.56
37	24.90	3,496	Erucic acid	$C_{22}H_{42}O_2$	0.69	1.25
38	26.14	16,470	Heptacosane	$C_{24}H_{50}$	0.72	1.36
39	27.750	16,410	Nonacosane	$C_{29}H_{60}$	39.40	48.89
40	28.60	8,867	Cholesta-4,6-dien-3-ol	$C_{27}H_{44}O$	0.87	1.85
41	29.46	8,867	4,6-Cholestadien-3-ol	$C_{27}H_{44}O$	1.66	3.69
42	29.53	4,462	Hentriacontane	$C_{44}H_{90}$	2.25	5.68
43	31.18	5,078	Ergost-5-en-3-ol	$C_{28}H_{48}O$	1.21	3.45
44	32.42	1,672	Stigmast-5-en-3-ol	$C_{29}H_{50}O$	2.47	6.78
45	33.35	87,270	Hop-22(29)-en-3-one	$C_{30}H_{48}O$	1.29	3.46
46	34.62	56,230	Stigmast-4-en-3-one	$C_{29}H_{48}O$	1.04	2.56

–, not detected.
[a] Kovats indices.
[b] Volatile extracted by SFE under optimized condition 1.
[c] Volatile extracted by SFE under optimized condition 2.

Subsequently, the chemical compositions of the extracts obtained under two different optimized extraction conditions were investigated by GC/MS. The results are shown in Table 4. Forty-six compounds were identified from the extract of extraction condition 1, and the total volatile was consisted of hydrocarbon (49.65%), aldehyde (16.66%), fatty acid (22.38%), terpene (9.04%) and little alcoholic. By contrast, 32 compounds were identified under extraction condition 2, in which hydrocarbon, aldehyde, fatty acid and terpene possessed 58.21%, 5.97%, 13.19% and 21.79%, respectively. It is obvious that the contents of the chemicals extracted under these two conditions were different. The contents of hydrocarbon and terpene increased by17.24% and 141.0% from conditions 1 to 2, but the decrease of aldehyde and fatty acid from conditions 1 to 2 were 64.66% and 41.06%, respectively. In the literature [20,21], only five compounds (undecanoic acid, tetradecanoic acid, pertedecanoic acid, hexadecanoid acid and linoleic acid) were identified from the volatile oils of *P. Villosa* Juss. Among those five kinds of fatty acid, tetradecanoic acid and hexadecanoid acid were detected in our study. Such huge differences between present and previous studies might be attributed to different extraction methods and separation conditions.

Oxidative stress is an important factor in the genesis of many diseases, from cancer to cardiovascular and degenerative diseases [39–41]. In order to protect the body against the consequences of oxidative stress, an efficacious approach united the action of a wide spectrum of antioxidants is better than the activity of a single antioxidant, and that antioxidants from natural sources have a higher bioavailability and therefore higher protective efficacy than synthetic antioxidants [42]. Different chemicals showed different antioxidant activity in our research. The stronger scavenging effects may be due to higher content of oxygenated compounds (aldehyde and fatty acid), but hydrocarbons and terpene are naturally inactive as shown by investigating the scavenging effects of two main compounds of the extracts, nonacosane and *n*-hexadecanoic acid. EC_{50} values against DPPH and $ABTS^+$ of *n*-hexadecanoic acid were 76.78

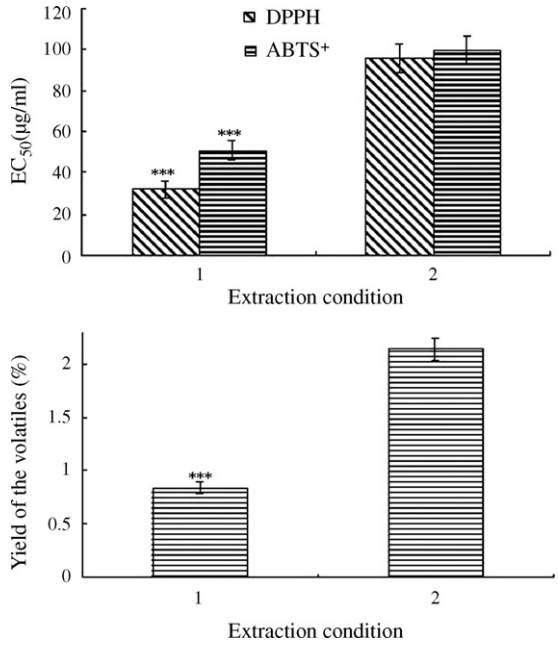

Fig. 3. The comparison between antioxidant activity and yield of the volatiles under two different optimal conditions. DPPH, 1,1-diphenyl-2-picrylhydrazyl; $ABTS^+$, 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt; EC_{50}, concentration of antioxidant required to quench 50% of the stable free radical; yield of the volatiles (%) = (the amount of the volatile (g)/the sample mass (g)) × 100%; extraction condition 1 = $A_3B_1C_2D_1$; extraction condition 2 = $A_2B_2C_3D_3$. ***, significant difference ($p < 0.001$) between 1 and 2 extraction conditions by Student's *t*-test ($n = 3$).

and 89.23 μg/ml, whereas nonacosane (belonging to hydrocarbons) was unable to scavenge the free radicals.

The volatiles are minor components in *P. Villosa* Juss. The flavonoids, with high content in *P. Villosa* Juss, have higher antioxidant activity than the volatiles. Although in our present study the volatile constituents were the research object, the EC_{50} values of non-volatile constituents, which were extracted by the customary method, have also been measured. The results show that the EC_{50} values against DPPH and $ABTS^+$ of the extract were 8.61 and 9.12 μg/ml, which were a little higher than those of ascorbic acid.

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