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RESEARCH ARTICLE

Supercritical fluid CO_2 extraction of essential oil from *Marchantia convoluta*: global yields and extract chemical composition

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Abbreviations: SFE: Supercritical fluid extraction
GC-MS: Gas chromatography-mass spectrometry

The essential oil of *Marchantia convoluta* was obtained by supercritical (carbon dioxide) extraction using methanol as a modifier. Global yields were determined according to the orthogonal design. The effects of different parameters, such as pressure, temperature, modifier volume and extraction time, on the supercritical fluid extraction (SFE) of essential oil from *M. convoluta* were investigated. Maximum global yields were obtained using the following conditions: extraction temperature, 35ºC; dynamic time, 35 min; pressure, 15 Mpa and modifier volume, 40 mL. The essential oil extract was analyzed by capillary gas chromatography with mass spectrometric detector (GC-MS). The compounds were identified according to their retention indices and mass spectra (EI, 70 eV). The results from GC-MS and literature were compared.

Marchantiaceae plants are well-known traditional Chinese medicinal herbs and extensively used to treat tumefaction of skins, protect liver, treat hepatitis and used as antipyretic in countryside (Chen and Xiao, 2005; Xiao et al. 2005a; Zhu et al. 2005). There are a large number of Marchantiaceae plants in Guangxi Zhuang Autonomous District such as *Marchantia polymorpha*, *M. convoluta* and *M. paleacea*. These species live in together and it is difficult to be distinguished one from the others because of their genetic similarity. *M. convoluta* was only found in China (Tian et al. 1999).

Compared to *M. polymorpha*, *M. convoluta* is quite rare and was thought of negligible by people many years ago. The major identified constituents in *M. convoluta* were flavonols, triterpenoids, and steroids (Cao et al. 2005; Chen and Xiao, 2005; Chen and Xiao, 2006; Xiao et al. 2005a; Xiao et al. 2005b; Xiao et al. 2006a; Zhu et al. 2005; Zhu et al. 2003). The flavonoids of *M. convoluta* mainly consist of quercetin, luteolin, apigenin and their O- and C-glycosides (Chen and Xiao, 2005; Xiao et al. 2005a; Xiao et al. 2005b;

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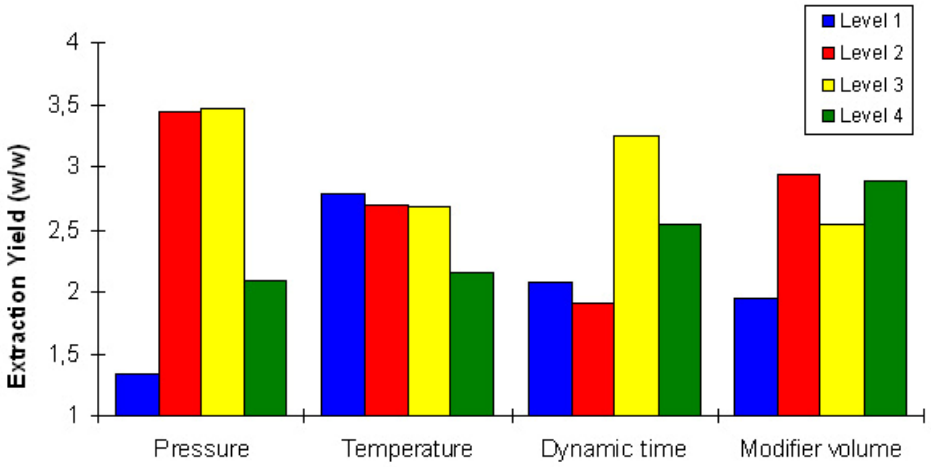

Figure 1. The yields of volatile oil under orthogonal conditions. For designations of factors pressure, temperature, dynamic time and modifier volume, see Table 1.

Zhu et al. 2005; Chen and Xiao, 2006; Xiao et al. 2006b). Dried leaves are used in China to protect livers and to treat tumefaction of skins. A high dosage of flavonoids from *M. convoluta* (20 and 40 µg/mL) could significantly reduce the activity of ALT (Alanine aminotranferease) and AST (Alanine aminotranferease) in the serum of mice with acute hepatic injury caused by CCl_4 and increase the contents of TP (Total protein) and ALP (Alkaline phosphatase), as well as inhibit the auricle tympanites of mice caused by dimethylbenzene. Flavonoids from *M. convoluta* can strongly inhibit colibacillus, tyhoid bacillus, Staphylococcus aureus, bacillus enteritidis, hemolytic streptococci type B and Diplococcus pneumoniae, and possess distinct effect of antibiosis, anti-inflammation and diuresis in mice (Xiao et al. 2005a). Moreover, flavonoids from *M. convoluta* has anti-hepatitis B Virus activity (Xiao et al. 2005b). Extracts from *M. convoluta* can also strongly inhibit tumors in human liver and lung cancer cell lines (Chen and Xiao, 2006; Xiao et al. 2006a).

The extraction of essential oil components using solvent at high pressure, or supercritical fluids (SCF), has received much attention in the past several years, especially in food, pharmaceutical and cosmetic industries, because it presents an alternative for conventional processes such as organic solvent extraction and steam distillation (Fekete et al. 1996; Assis and Lanças, 1999; Doraiswamy et al. 1999; Eikani et al. 1999). Supercritical fluid extraction allows a continuous modification of dissolution power and selectivity by changing the solvent density. It has the density of a liquid and solubilizes solids like a liquid solvent, but has a diffusion power similar to a gas and permeates through solid materials very easily. The power of solubilization increases with the density of the fluid; high densities of a supercritical fluid are possible at high pressures and allow it to dissolve large quantities of organic compounds. The dissolved compounds can be recovered from the fluid by reduction of its density, by means of decreasing the pressure or increasing the temperature. This low temperature separation process prevents the degradation of the chemical compounds of the extract due to heat, as in steam distillation (Anitescu and Doneanu, 1998; Lanças and Sargenti, 1998; Gamiz-Gracia and Luque, 2000; Michielin et al. 2005; Raeissi and Peters, 2005; Sovová, 2005).

An essential drawback in the use of supercritical CO_2 is its low polarity, making the extraction of polar analytes difficult. Nevertheless, this limitation may be overcome by adding small amounts of polar modifiers, such as methanol or ethanol to the supercritical CO_2, in order to increase its solution power. In the present work, the modifier methanol enhanced the solubility of solutes in supercritical CO_2 and thus the efficiency of extraction increased.

SFE appears to be a cost-effective technique in laboratory scale, but an accurate economic evaluation for large-scale units requires supplementary experiments. The advantages of SFE-CO_2 extraction over the petrol ether extraction include: low operating temperature, hence no thermal degradation of most of the labile compounds; shorter extraction period; high selectivity in the extraction of compounds; no solvent residue with negative effects on the oils quality. The essential oils of plants have usually been isolated by either hydrodistillation or solvent extraction. The disadvantages of all these techniques are: low yield, loss of volatile compounds, long extraction time, toxic solvent residues and degradation of unsaturated compounds, giving undesirable off-flavour compounds, due to heat.

The aim of the present work is to investigate the effects of different parameters, such as pressure, temperature, modifier volume and dynamic extraction time, on the supercritical fluid carbon dioxide extraction of *M.*

Table 1. The results of orthogonal test $L_{16}(4^4)$.

Expt No.	Pressure (MPa)	Temperature (ºC)	Dynamic time (min)	Modifier volume (ml)	Extraction yield (%, w/w)
1	5	35	25	30	0.99
2	5	45	15	40	0.87
3	5	55	45	10	1.34
4	5	65	35	20	2.12
5	10	35	15	10	2.68
6	10	45	25	20	3.27
7	10	55	35	30	3.81
8	10	65	45	40	4.01
9	15	35	35	40	4.69
10	15	45	45	30	4.23
11	15	55	15	20	3.56
12	15	65	25	10	1.38
13	20	35	45	20	2.79
14	20	45	35	10	2.41
15	20	55	25	40	2.00
16	20	65	15	30	1.16

convoluta. To the best of our knowledge, no report has yet appeared on the SFE of the plant species.

MATERIALS AND METHODS

Plant materials

The whole plants of *Marchantia convoluta* were collected in Shangling City of Guangxi Zhuang Autonomous District in August, 2003. The specimen (No 20041364) was identified by Zhou Zi-jing, at Biology Department of Guangxi Chinese Medical University. The dried leaves were stored in dark at 4ºC for 20 days. Immediately prior to the extraction process, the leaves were ground in a blender to produce a powder with an approximate size of 0.4 mm.

Reagents

HPLC grade methanol and analytical grade petroleum ether were purchased from Hanbon Company Limited. Carbon dioxide (99.99% purity) contained in a cylinder with an eductor tube, was obtained from CSU Co. (Changsha, China).

Supercritical fluid extraction (SFE)

A Suprex MPS/225 system (Pittsburgh, PA) in the SFE mode was used for all the extractions. The extraction vessel was a 10 mL stainless steel vessel. Supercritical fluid extractions were conducted at pressures of 5, 10, 15 and 20 MPa and temperatures of 35, 45, 55 and 65ºC for a duration of 20 min, in static mode, followed by 15, 25, 35, or 45 min, in dynamic mode. A Durafow manual variable restrictor (Suprex) was used in the SFE system to collect the extracted analytes. In order to prevent sample plugging, the restricting point was warmed electrically. The supercritical CO_2 flow rate through the Durafow restrictor was approximately 0.3-0.4 mL/min (compressed). Plant powder (3.0 g) was well mixed with 2 mm diameter glass beads, and was then charged into the 10 mL extraction vessel. The essential oil was extracted from the plant using

supercritical CO_2 under various conditions according to the Taguchi method. Table 1 shows the experimental conditions for each of the SFE runs. The extracted oil was collected in dichloromethane in a 5.0 mL volumetric flask and the final volume of the extract was adjusted to 5.0 mL with dichloromethane at the end of the extraction. In order to improve the collection efficiency, the 5.0 mL volumetric flask was placed in an ice bath during the dynamic extraction stage. For all the modifier studies, methanol was spiked directly into the extraction vessel with charged sample prior to the extraction.

Four millilitres of solution were poured into a 20 mL beaker. The solvent was evaporated by bubbling argon gas through the solution. Then the weight of essential oil was measured and the extraction yield was calculated.

Gas chromatography-mass spectrometry

GC analyses were performed using a Shimadzu GC-9A gas chromatograph equipped with a FID and a HP-5 fused silica column (60 m x 0.25 mm i.d., 0.25 μm). Oven temperature was programmed 60ºC for 5 min, and then increased to 250ºC at a rate of 5ºC/min. Injector and

Table 2. GC-MS analytical results of SFE extract (No.9). The compounds were listed in order of elution time.

No	Compounds	Formula	RT /min	Relative Content (%)	Match (%)
1	Benzenamine, hydrochloride	C_6H_8ClN	3.239	0.608	93
2	2-ethyl-Hexanoic acid	$C_8H_{16}O_2$	4.594	9.821	96
3	Benzoic Acid	$C_7H_6O_2$	5.154	1.544	99
4	Methyl Salicylate	$C_8H_8O_3$	5.697	0.975	95
5	Benzothiazole	C_7H_5NS	6.131	11.822	95
6	2-Propenoic acid, 2-methyl-, 1,2-ethanediyl ester	$C_{10}H_{14}O_4$	7.092	0.143	87
7	2-(formyloxy)-1-phenyl-Ethanone	$C_9H_8O_3$	7.943	0.469	91
8	1-phenyl-1,2-Propanedione	$C_9H_8O_2$	8.280	1.733	90
9	6,6-dimethyl-Bicyclo[3.1.1]hept-2-ene-2-ethanol	$C_{11}H_{18}O$	8.492	0.107	91
10	1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-Naphthalene	$C_{15}H_{24}$	8.560	0.220	96
11	6,7-dihydro-3-hydroxy-1,8(2H,5H)-Isoquinolinedione	$C_9H_9NO_3$	8.960	0.374	88
12	1,4-dimethyl-3,3'-bis-Piperazine-2,5-dione	$C_{12}H_{18}N_4O_4$	9.029	0.594	87
13	(1-Propoxy-pentyl)-cyclopropane	$C_{11}H_{22}O$	9.818	0.103	97
14	Diethyl Phthalate	$C_{12}H_{14}O_4$	10.606	0.881	94
15	4 -methyl-myo-Inositol	$C_7H_{14}O_6$	10.801	1.392	98
16	Cedrol	$C_{15}H_{26}O$	10.864	4.604	94
17	2,4,6-trimethyl-Decane,	$C_{13}H_{28}$	11.121	0.102	93
18	2(3H)-Benzothiazolone	C_7H_5NOS	11.487	2.792	95
19	2,6-dimethyl-Heptadecane	$C_{19}H_{40}$	11.755	0.400	89
20	ethylphenoxy-Benzene	$C_{14}H_{14}O$	12.075	8.991	84
21	[3.3.1]nona-2,4-dione-9,9-Dimethoxybicyclo	$C_{11}H_{16}O_4$	12.338	0.446	89
22	1,1'-(3-methyl-1-propene-1,3-diyl)bis-Benzene	$C_{16}H_{16}$	12.733	2.070	90
23	Eicosane	$C_{20}H_{42}$	12.841	0.210	94
24	9-methylene-9H-Fluorene	$C_{14}H_{10}$	12.893	0.119	90
25	2,6,10-trimethyl-Dodecane	$C_{15}H_{32}$	12.973	0.203	89
26	3-phenyl-4-Isoxazolamine	$C_9H_8N_2O$	13.047	0.232	89

27	2-(1-phenylethyl)-Phenol	$C_{14}H_{14}O$	13.087	0.510	90
28	3-Eicosyne	$C_{20}H_{38}$	13.373	0.133	96
29	4-Cyanothiophenol	C_7H_5NS	13.464	5.492	86
30	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	13.899	0.665	88
31	4-Hydroxy-4-(2,6-dimethylcyclohex-3-enyl)butan-2-one	$C_{12}H_{20}O_2$	14.327	0.472	90
32	1,2-Benzenedicarboxylic acid, bis(1-methylethyl) ester	$C_{14}H_{18}O_4$	14.642	0.148	91
33	14-methyl-Pentadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	14.716	0.257	98
34	Diphenyl sulfone	$C_{12}H_{10}O_2S$	14.984	1.033	93
35	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	15.316	2.080	95
36	Dibutyl phthalate	$C_{16}H_{22}O_4$	15.510	1.336	92
37	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	16.025	1.447	91
38	2-Nonadecanone	$C_{19}H_{38}O$	18.362	0.332	90
39	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	18.791	0.315	99
40	(Z,Z)-9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	18.894	1.311	96
41	13-Tetradecenal	$C_{14}H_{26}O$	18.997	1.292	96
42	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	19.511	3.254	89
43	Ethyl Oleate	$C_{20}H_{38}O_2$	19.625	1.601	86
44	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	20.134	2.386	86
45	Acetic acid, octadecyl ester	$C_{20}H_{40}O_2$	20.425	8.817	93
46	1,3-Diphenyl-1-(2-hydroxyphenyl)butane	$C_{22}H_{22}O$	24.660	0.333	97

detector temperatures were 250 and 265ºC, respectively. The carrier gas, helium, was adjusted to a linear velocity of 30 cm/s. The SFE samples (1 µL) were injected into GC/MS (without any further dilution) using the split mode with a split ratio of 1/60. Petrol ether extraction extract was diluted 30 times and 1 µL of the diluted solution was injected into GC with the same split ratio. The ionization energy was 70 eV with a scan time of 1 sec and mass range of 40-540 amu. The percentages of compounds were calculated by the area normalization method without considering response factors. The components of the oil were identified by comparison of their mass spectra with those of a computer library or with authentic compounds. Data obtained were conformed by comparison of their retention indices, either with those of authentic compounds or with data published in the literature (Sandra and Bicchi, 1987).

RESULTS AND DISCUSSION

Optimization of the experimental conditions

Since various parameters potentially affect the extraction process, the optimization of the experimental conditions represents a critical step in the development of a SFE method. In fact, pressure and temperature of the fluid, percentage of the modifier and the extraction times are generally considered as the most important factors. The optimization of the method can be carried out step-by-step or by using an experimental design. Table 1 shows different conditions of experiments carried out with SFE for extractions of *M. convoluta* according to the Taguchi experimental design. All the selected factors were examined using a four-level orthogonal array design with an $L_4$16 (4^4) matrix. In general, a full evaluation of the effect of four factors from three levels on the yield needs 256 (4^4) experiments. In order to reduce the number of experiments, a L_4 (4^4) orthogonal design graph was used (Table 1), reducing the number of experiments to 16. The yields obtained under orthogonal conditions are also shown in Table 1. The extraction yields were 0.87% - 4.69%.

In this study, interactions among variables were not incorporated in the matrix and focus was placed on the main effects of the four most important factors. The results of the SFE experiments, based on extraction yields, are given in Table 1.

The mean values of the extraction yields for the corresponding factors at each level were calculated according to the assignment of the experiment (Figure 1). For example, the extraction yields of the four trials at 15

MPa were evaluated as mean values of the corresponding four runs. The mean values of the four levels of each factor (*e.g.*, pressure) reveal how the extraction yield changes when the level of that factor is changed. Figure 1 shows the variations in extraction yield as a function of change in different levels of the factors studied. For the complete recovery of the main components of the plant, higher pressures are necessary. This is because raising the extraction pressure at constant temperature leads to higher fluid density, which increases the solubility of the analytes. To obtain quantitative recovery of analytes, they must be efficiently partitioned from the sample matrix into the supercritical fluid. The influence of temperature on the composition of the extracts was studied. Higher temperature resulted in lower extraction yield. Higher temperature can decrease fluid density and thus reduce extraction efficiency. For all the analytes, the volume of the modifier was found not to be a significant parameter. The influence of the dynamic extraction time on the composition of the extracts was studied. Extraction was performed with supercritical carbon dioxide at the static extraction step of 20 min, followed by 15, 25, 35 and 45 min of dynamic extractions. Results showed that increasing dynamic extraction time to 35 min enhanced the extraction of most components. Thus, the best conditions, obtained by preliminary test, for the extraction of oil were: extraction temperature: 35ºC; dynamic time, 35 min; pressure, 15 Mpa and modifier volume, 40 mL.

GC-MS analyses

The compounds from the oil produced by SFE using no. 9 orthogonal test conditions were identified and quantified by GC-MS (Table 2). The total ion chromatograph of SFE using Expt no. 9 orthogonal test condition was shown in Figure 2. GC separation gave 50 peaks, among which 46 were identified by MS library matching. The peak area of compounds identified accounted for 84.16% of total peak area.

The major compounds identified in SFE extract no. 9 were: benzothiazole (11.82%), 2-ethylhexanoic acid (9.82%), ethylphenoxybenzene (8.99%), acetic acid octadecyl ester (8.82%), 4-cyanothiophenol (5.49%), cedrol (4.60%), 9,12-octadecadienoic acid ethyl ester (3.25%), 2(3H)-benzothiazolone (2.79%), octadecanoic acid ethyl ester (2.39%), n-hexadecanoic acid (2.08%), 1,1'-(3-methyl-1-propene-1,3-diyl) bis-benzene (2.07%). The total content of

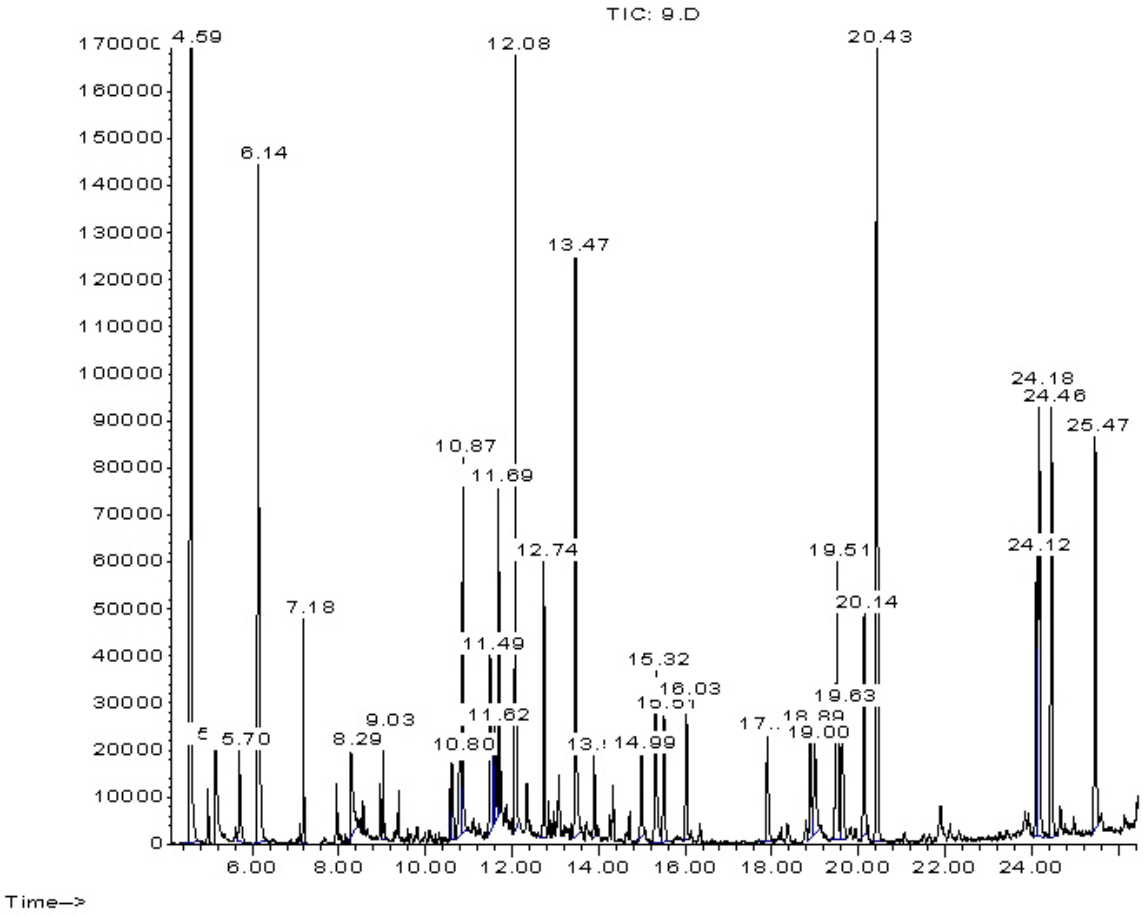

Figure 2. Total Ion Chromatograph of SFE extract (no. 9).

organic acids and esters was 32.19%.

Extraction of natural products by different methods may yield different chemical components (Stashenko et al. 1996; Kohler et al. 1997; Vinatoru, 2001; Kim and Lee, 2002; Pourmortazavi et al. 2003; Lucchesi et al. 2004; Menaker et al. 2004; Seger et al. 2004; Braga et al. 2005; Fulzele et al. 2005; Michielin et al. 2005; Sporring et al. 2005). Several studies on compositions of the extract from *M. convoluta* were reported (Zhu et al. 2003; Cao et al. 2005; Chen and Xiao, 2005; Xiao et al. 2005a; Xiao et al. 2005b; Zhu et al. 2005; Chen and Xiao, 2006). Zhu et al. (2003) separated β-sitosterol and stigmasterol from the methanol extract. Chen and Xiao (2005) separated and determined flavonoids of *M. convoluta* by RP-HPLC. Cao et al. (2005) extracted bioactive components from *M. convoluta* with 80% ethanol. The extract was suspended in water and extracted with petroleum ether, EtOAc and n-BuOH successively. The petroleum ether extract and EtOAc extract were analyzed by capillary gas chromatography with mass spectrometric detector (GC-MS) (Cao et al. 2005). The results were different from each other because of different methods dealing with the extract. As shown in the Table 2 and discussed by Cao et al. (2005), the composition of the SFE products and the extracts extracted by petrol ether and ethyl acetate are different. Higher levels of ester (accounting for 57.21%) were found in the extracts extracted by petrol ether while higher levels of terpenes and derivatives were found in the SFE product. The benzothiazole content in the SFE extract is considerable (11.82%) and the organic acids and esters accounted for 32.19%. This is similar to the report by Cao et al. (2005). On the other hand, Cao et al. (2005) reported that higher benzothiazole content (14.97%) in the ethyl acetate extract while organic acids and esters accounted for 36.01% in the petrol ether extract. Cao et al. (2005) also reported that a phytol content of 6.32% in the petrol ether extract, whereas it was not found in the SFE products.

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