Characterization of Neem (*Azadirachta indica* A. Juss) seed volatile compounds obtained by supercritical carbon dioxide process

R. Swapna sonale¹ · K. Ramalakshmi¹ · K. Udaya Sankar¹

Revised: 9 January 2018 / Accepted: 26 January 2018 / Published online: 3 February 2018
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Abstract Extraction process employing Supercritical fluid carbon dioxide (SCF) yields bioactive compounds near natural forms without any artifact formation. Neem seed was subjected to SCF at different temperatures and pressure conditions. These extracts were partitioned to separate volatile fraction and were analyzed by Gas Chromatography–Mass spectroscopy along with the volatiles extracted by the hydro-distillation method. Experimental results show that there is a significant effect of pressure and temperature on isolation of a number of volatile compounds as well as retention of biologically active compounds. Twenty-five volatile compounds were isolated in the Hydro-distillate compare to the SCF extract of 100 bar, 40 °C which showed forty volatile compounds corresponds to 76.38 and 92.39% of total volatiles respectively. The majority of bioactive compounds such as Terpinen-4-ol, 1,2,4-Trithiolane, 3,5-diethyl, allyl isopropyl sulphide, Cycloisolongifolene, á-Bisabolene, (-)-α-Panasinsen, Isocaryophyllene, trans-Sesquisabinene hydrate, 1-Naphthalenol, were identified in the extract when isolated at 100 bar and 40 °C.

Keywords *Azadirachta indica* · Neem seed · Kernel · Volatile · Supercritical fluid carbon dioxide extraction

Introduction

Neem (*Azadirachta indica* A. Juss) tree belongs to the family of Meliaceae. Neem is one of the most versatile medicinal plants having a broad spectrum of biological activity in India and its neighboring countries since immemorial. Neem seed oil and extract are proved effective against some fungi that cause infection to human body. It is used as anthelmintic, antileprotic (prevents leprosy), antibacterial, antidermatophytic activity. Neem seed spent (cake) is an active ingredient in preparation of mosquito repellent coil. A bitter compound, nimbidin is isolated from seeds which is effective in reducing fasting blood glucose (Biswas et al. 2002). The different parts of neem (seeds, leaves, flowers and bark) are used as traditional medicine for the household remedy against various human ailments. It also used as the natural pesticide, a raw material for cosmetic industry and other commodities (Ghimeray et al. 2009; Rashid et al. 2004; Olabinri et al. 2009; Aromdee and Sriubolmas 2006). Neem seed kernels constitute 50–60% of the seed and the remaining is the kernel portion. The fat content of the kernels ranges from 33 to 45%. Neem seed contains 40–45% of oil and remaining is the cellular matrix of the seeds. The oil is brownish yellow, non-drying oil with an acrid taste and unpleasant odor (Johnson and Morgan 1997). Several valuable bioactive substances from limonoids class of triterpenoids were found in neem oil. Azadirachtin (Azadirachtin A), Nimbidin, 3-tigloylazadirachtol (Azadirachtin B), Salannol, Salannin, Nimbinin, Nimbin, Nimbidin, and 1-tigloyl-3-acetyl-11-hydroxymeliacarpin (Azadirachtin D) non-volatile compounds are the major once (Mongkholkhajornsilp et al. 2005; Morgan 2009; Melwita and Ju 2010). Further, sulfur modified fatty substance like loeic acid (50–60%), palmitic acid (13–15%), stearic acid (14–19%),

✉ K. Ramalakshmi
ramal_kumar@yahoo.com

¹ CSIR- Central Food Technological Research Institute,
Mysore 570020, India

linoleic acid (8–16%) and arachidic acid (1–3%) are present in neem seed oil.

Volatiles isolated from neem seed oil also play a vital role in health care systems of the most traditional and modern medicines. By head space analysis insect repellent active compounds such as caryophyllene oxide, limonene, linalool, 1-hexanol and organosulphur compounds were identified from neem seed volatiles.

Hydro-distillation is a traditional technique for extraction of volatile oil. This distillation has several disadvantages, such as incomplete extraction of volatiles from plant materials, high operating temperatures with the consequent breakdown of thermally labile components, promotion of hydration reactions of chemical constituents, hydrolysis and solubilization in water of some compounds that alter the flavor and fragrance profile of many essential oils extracted by this technique. It also requires a post extraction process to remove water (Metherel et al. 2009). The other problem with this method is the production of waste water with a high biological oxygen demand (BOD). With high BOD the release of waste water is not permitted in many countries, again which requires an expensive installation of water treatment facility (O'shea et al. 2001).

In recent decades, the supercritical fluid carbon dioxide extraction (SCF-CO₂) has received special attention in extraction of volatile oil from natural materials. In compressed carbon dioxide (CO₂) main volatile oil constituents like hydrocarbon and oxygenated mono- and sesquiterpenes are solubilized (Stahl and Gerard 1985). In fact, CO₂ is physiologically harmless, non-toxic and non-corrosive. It readily reaches critical temperature (31.1 °C) and pressure (73.8 bar). Very little work was traced about the essential oil of neem seed by Supercritical fluid extraction. The present study was undertaken to employ supercritical carbon dioxide to extract the volatiles from neem seed and characterization of the constituents.

Materials and methods

Chemicals and reagents

Analytical grade chloroform procured from Merck (Mumbai, India). Carbon dioxide (purity 99.9%) was obtained from M/S Kiran Corporation, Mysore, India. Homologous series of C₈-C₂₂ n-alkane standards were procured from Sigma (St. Louis, MO, USA). Anhydrous sodium sulfate was obtained from SLR Chemicals (Mumbai, India). All other chemicals, solvents, and reagents used were of analytical grade.

Plant material

Neem seeds were purchased from a local market at Mysore, Karnataka, India. The seeds were manually cleaned to remove the adhering soil and extraneous matter if any. These cleaned neem seeds were dried in cabinet tray drier (M/S Armstrong Smith, India) at 40 °C for 24 h. Further, neem seed was dehulled by using DIAF huller (Crompton Parkinson Ltd, Bombay). Dehulled neem seed was ground by using hammer mill (Model: CIS-100, M/s Galai Production, Israel) to get powder of 32 mesh size. The moisture content of neem seed powder is $16 \pm 0.1\%$.

Supercritical fluid carbon dioxide extraction and partitioning of flavor volatiles

Extractions were carried out in NOVA Swiss equipment (WERKEAG, EX 1000, 1.4–1.2 V type, Switzerland) designed for working pressure of 1000 bar and temperature of 100 °C. Neem seed powder (300 g) was loaded into a steel cylinder equipped with sintered metal plate on both ends. The cylinder was then introduced into the extraction vessel, and required extractor and separator temperatures were maintained using thermostat water circulators. CO₂ supplied from a gas cylinder was compressed by using two diaphragm compressors to the desired pressure by adjusting the pressure controller and heated to the specified temperature using a heat exchanger to reach the supercritical state (Zarena et al. 2012). Nine experiments were carried out in duplicates at temperatures of 40, 50 and 60 °C; the pressure of 100, 150 and 200 bar (Table 1). After completion of an experiment, the extract was collected from the separator and stored at 4 °C for further study.

All the SCF extracts were further hydro-distilled, and the distillate was partitioned with chloroform to separate the volatiles. Chloroform was removed using a rotavapor (RE111, Buchi, Switzerland) and pale yellow colored oil obtained was dried over anhydrous sodium sulfate. The flavor isolate was stored at 4 °C for further study.

Extraction of volatile oil by hydro-distillation (Conventional method)

Neem seed powder (250 g) was defatted by Soxhlet extraction using petroleum ether (60–80 °C) with 15–16 siphons in 12 h. Solvent adhering to the Marc was expelled by a screw press. Further, neem seed powder was dried in the sun for 2 h and again dried in an oven at 105 °C for 2 h. Defatted neem seed powder was subjected to hydro-distillation in Clevenger distillation until all the oil is collected (9 h). The pale brown colored oil obtained was dried over anhydrous sodium sulfate and stored at 4 °C for

Table 1 Yield of SCF extracts in different conditions

No of SCF experiments	Yield ^a (g/100 g)	Yield ^a of Volatiles (mg/100 g)
100 bar-40 °C	4.61	24
100 bar-50 °C	4.62	39
100 bar-60 °C	1.2	15
150 bar-40 °C	17.11	49
150 bar-50 °C	10.72	35
150 bar-60 °C	10.99	19
200 bar-40 °C	31.57	162
200 bar-50 °C	18.43	68
200 bar-60 °C	21.39	67

^aYield was given in raw material basis

further analysis. All the experiments were executed in triplicate, and the result was expressed.

Chemical characterization of volatile oil and chromatographic analysis

Gas chromatography–mass spectrometry (GC–MS) analysis

The volatile oil was characterized using Perkin Elmer GC equipped with quadrupole mass spectrometer (Model: Turbomass Gold) fitted with an Elite-5 fused silica column (30.0 m × 250 μm film thickness) coated with polydimethylsiloxane (PDMS). Initially, the oven temperature was held at 35 °C for 2 min, ramp 2 °C/min to 70 °C, held for 0 min then increased at 4 °C/min to 250 °C, held for 5 min at final temperature. The carrier gas (helium) was delivered with 75 kPa inlet pressure with linear velocity (20 cm/s), injector temperature (180 °C) and detector temperature (230 °C). Samples were diluted with chloroform, and 1 μl was injected. The mixture of n-alkanes as standards (C8–C24) diluted with chloroform was also injected and analyzed at same conditions to determine the Kovats' retention indices by the following equation.

$$KI = 100[n + (N - n) \times \{\log \text{tr}(\text{unknown}) - \log \text{tr}(n)\} / \{\log \text{tr}(N) - \log \text{tr}(n)\}]$$

where (n) is stand for the number of carbon atoms in the shorter chain alkane, N is the number of carbon atoms in the longer chain alkane, (tr) is the adjusted retention times for unknown compounds, tr (n), shorter-chain and tr (N), longer-chain alkanes. The total volatile production was estimated as the sum of all GC-FID peak areas in the chromatogram, and individual compounds were quantified as relative percent area. All data are means of three independent determinations expressed as the percentage of total peak area (Shivakumar et al. 2012).

Results and discussion

Supercritical fluid extraction has increased during two last decades in the extraction of plant volatile components since it is a rapid, selective and convenient method. Food grade Carbon dioxide (GRAS) which is used in SCF is an excellent alternative for chemical solvents (Pourmortazavi and Hajimirsadeghi 2007). In general, the extractability of volatile and non-volatile compounds by SCF depends on the occurrence of functional groups, molecular mass and polarity. Essential oil of coriander seeds was extracted by SCF under different conditions of temperature and pressure by Grosso et al. (2008). GC–MS analysis of volatile oil composition of SCF extract reveals that the best condition was achieved at 90 bar and 40 °C.

Extraction efficiency by evaluating SCF parameters

Neem seed (39 kg) with the moisture content of 17% was dehulled to get the kernels of 16 kg which possess the moisture content of 19.3%. The dehulled seed was ground and used for further experiments.

Supercritical fluid extraction has attracted a vast deal of attention. Indeed, it is an excellent alternative method for seed oil extraction to replace classical industrial methods. It becomes the focus of attention due to its chemical and physical properties. Furthermore, it offers recovery of solvent-free analyte from solid or semi-solid samples that are equal to or better than that of conventional extraction process (Herrero et al. 2010). CO₂ is environmentally friendly and recognized as safe. It is also essential for the sample preparations of food and natural product. SCF using CO₂ can be operated at low temperatures using a non-oxidant medium, which allows the extraction of thermally labile or easily oxidized compounds. In this study, the volatile composition of neem seed at three pressures (100, 150 and 200 bar) and three temperatures (40, 50 and 60 °C) in different combination was studied. And also,

SCF volatile extract was compared with conventional hydro-distillation process.

In SCF, totally nine experiments conducted in duplicates and the results were analyzed. The method of extraction usually has a significant effect on the quality and quantity of the volatile oils by varying temperature and pressure in SCF extraction. The extraction yields of nine experiments were evaluated, the factor (e.g., pressure) reveal how the extraction yield changes when the level of that factor is changed. As per the earlier report (Mostafa et al. 2004), an increase in extraction pressure, at a constant temperature, leads to higher fluid density. This higher fluid density increases the solubility of the analytes.

The effect of temperature on the yield as well as the composition of extracts was evaluated. Higher temperature decreased fluid density and thus reduced extraction efficiency. When extraction was done at the different pressure of 100, 150 and 200 bar, the yield of volatile extract was more at 40 °C (Table 1). Further, it was observed that the volatiles percentage is directly related to the pressure and temperature, in turn, the density of carbon dioxide. With increasing temperature, at the pressure of 100 bar, the extract yields decreased drastically, but there is an increase in the volatiles percentage in the extract. But, at 150 and 200 bar higher pressures, there is definite increase in the volatiles percentage but not perceptible, as the total extract yields increased and the solubilization of fatty oil as well as high molecular compounds with increased density of carbon dioxide. In the extraction of pepper oil, this relationship on temperature, pressure density of volatiles correlates the earlier publications (Sankar 1989). The conventional method of hydro-distillation has been traditionally used for the extraction of volatile oils on a laboratory scale. In this study, the volatile composition of neem seed obtained by SCF extraction was compared with the conventional extraction. Further, the conventional hydro-distillation of neem seed volatile extract yielded 33 ± 0.76 mg/100 g after the removal of 53% of fat.

GC–MS analysis

Biogenetically, most of the volatile compounds are derived from mevalonic acid (MVA) pathway (i.e., triterpenoids and sesquiterpenoids). However, monoterpenoids, diterpenoids, and tetraterpenoids are biosynthesized via the plastidial methylerythritol phosphate (MEP) (Sawai and Saito 2011). In general, mono and diterpenes are more soluble than paraffin and fatty acids at low CO₂ densities (T = 40–50 °C, p = 80–90 bar), whereas sesquiterpenes and oxygenated terpenes are soluble at high CO₂ densities (T = 40–50 °C, p = 100–200 bar). The volatile neem isolates were subjected to GC–MS analysis. The chemical

components present in the volatile oil were identified according to their order of elution on Elite-5 column.

All nine SCF experiment results were analyzed by GC–MS. At 100 bar, 40 °C forty compounds were identified, which represented 92.39% of total volatiles. Similarly, at 100 bar, 50 °C thirty-two compounds (85.9%), 100 bar, 60 °C twenty-two (56.01%), 150 bar, 40 °C thirty-three (73.34%), 150 bar, 50 °C thirty-seven (70.52%), 150 bar, 60 °C thirty-three (76.87%), 200 bar, 40 °C twenty-seven (65.71%), 200 bar, 50 °C nine (72.78%), 200 bar, 60 °C twenty three (52.47%) were identified respectively. The results of SCF volatile compounds are compiled in Table 2 (SCF 100 bar at 40, 50 and 60 °C), Table 3 (SCF 150 bar at 40, 50 and 60 °C) and Table 4 (SCF 200 bar at 40, 50 and 60 °C). Further, the hydro-distillation of neem seed volatile extract is 33 mg/100 g. Thirty-two compounds were identified which represent the 76.38% of the total volatile extract.

The major biologically active compounds of 100 bar, 40 °C of SCF extract are Terpinen-4-ol (0.44%), 2, 4-Decadienal (0.85%) (Zai-Chang et al. 2005), α -Cedrene (0.70%), Bicyclosesquiphellandrene (1%) (Aneja et al. 2005), Bicyclo [3.1.1] hept-2-ene, 2, 6-dimethyl-6-(4-methyl-3-pentenyl)- (2.1%) (Liu et al., 2011), Allyl isopropyl sulfide (2.9%) (Muhi-Eldeen et al. 2008), β -Sesquiphellandrene (0.37%) (Jayaprakasha et al. 2005), Cycloisolongifolene (0.49%) (da Silva et al. 2015), Guaia-1(10), 11-diene (3.42%), β -Bisabolene (3.27%), (–)- α -Panasinene (0.36%), Isocaryophyllene (0.41%), trans-Sesquisabinene hydrate (0.39%), 1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1 α , 4 α , 8 α)]- (5.1%), Hexadecanoic acid, methyl ester (3.95%), n-Hexadecanoic acid (23.66%), and Cis-Vaccenic acid (2.99%) (Huang et al. 2011). These above compound are biologically active as antifungal, anti-influenza, antimicrobial, and antioxidants. On the other hand, the majority of these compounds are absent in hydro-distillation of neem volatiles. Hydro-distilled neem seed volatile mainly comprises of 1,2,4-Trithiolane, 3,5-diethyl (43.91%), cycloeicosne (3.57%), n-Tetracosanol-1 (3.13%), E-15-Heptadecenal (4.07%), 1-Hexadecanol (4.51%) and others (Table 5). As shown by the results, the composition of the SCF and hydro-distillation was significantly different.

It is found that in all nine experiments of SCF, higher levels of compounds having carboxylic acid functional groups (25–65%). At 100 bar, 40 °C, the volatile compounds were further classified as acids (30%), alcohols (6.42%), aldehyde (0.857%), alkane (0.46%), alkene (19.48%), ester (13.93%), lactone (0.35%), n-heterocycle (0.41%), oxime (0.57%), s-heterocycle (1.05%) and α , β -unsaturated ketone (1.45%). The presence of compounds of these entire functional groups make 100 bar, 40 °C is the suitable condition for extraction of neem seed volatile in

Table 2 Identification of neem seed (100 bar, 40, 50 and 60 °C) volatile compounds by GC–MS

Sl. no	RT (min)	Compound	100 bar (ppm)			RI
			40 °C	50 °C	60 °C	
1	16.873	Butanal, oxime	1.69	–	–	1020
2	25.577	Pyrazine, 3,5-diethyl-2-methyl-	1.21	–	–	1170
3	26.562	Terpinen-4-ol	1.28	–	–	1189
4	27.423	Octanoic acid	4.08	2.26	–	1207
	27.563					1210
5	31.044	Nonanoic acid	2.94	2.57	–	1297
	31.209					1301
6	31.969	2, 4- Decadienal	2.50	0.77	–	1323
	31.969					
7	32.2	1,2,4-Trithiolane, 3,5-diethyl-	1.51	–	–	1330
8	33.72	2(3H)-Furanone, dihydro-5-pentyl-	1.02	0.06	–	1373
	33.72					
9	35.146	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3à,3aà,7à,8aà)]-	1.14	–	–	1414
10	35.276	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	1.73	–	–	1418
11	35.421	Cedrene	2.06	–	–	1423
12	35.841	(+)-epi-Bicyclosquiphellandrene	2.952	–	–	1436
13	35.971	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	6.29	–	–	1440
14	36.066	1,2,4-Thiadiazole-3,5-diamine	–	–	11.23	1443
15	36.081	1-Propene, 3-[(1-methylethyl)thio]- (or) Allyl isopropyl sulfide	8.71	8.61	–	1444
	36.111					1445
16	36.251	4,7-Methanoazulene, 1,2,3,4,5,6,7,8-octahydro-1,4,9,9-tetramethyl-, [1S-(1à,4à,7à)]-	1.23	–	–	1449
17	36.381	γ-Murolene	2.53	–	–	1453
18	36.656	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*,S*)]-	1.10	–	–	1461
19	36.986	Spiro[4.5]dec-7-ene, 1,8-dimethyl-4-(1-methylethenyl)-, [1S-(1α,4β,5α)]-	5.88	–	–	1472
20	37.527	Cycloisolongifolene	1.43	–	–	1488
21	37.847	Guaia-1(10),11-diene	10.2	–	–	1498
22	38.327	à-Bisabolene	9.57	–	–	1514
23	38.602	(-)-α-Panasinsen	1.06	–	–	1523
24	39.127	Dodecane, 4,6-dimethyl-	–	–	1.78	1541
25	39.332	Isocaryophyllene	1.22	–	–	1548
26	40.293	Dodecanoic acid	1.13	–	–	1580
27	40.643	4,6-Diethyl-1,2,3,5-tetrathiolane	1.56	–	–	1591
	40.753					1595
28	40.833	2-(2-Isopropenyl-5-methyl-cyclopentyl)-acetamide	–	0.68	–	1598
29	42.613	trans-Sesquisabinene hydrate	1.90	0.76	–	1661
	42.614					
30	42.899	Cedrane or 1H-3a,7-	1.35	0.82	–	1671
	42.899	Methanoazulene, octahydro-3,6,8,8-tetramethyl-, [3R-(3à,3aà,6à,7à,8aà)]-				
31	43.349	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1α,4β,8α)]-	14.94	10.13	–	1687
	43.424					1690
32	43.684	4-(1,5-Dimethylhex-4-	1.89	1.40	–	1699
	43.689	enyl)cyclohex-2-enone				
33	43.794	2,4a-Methanonaphthalen-7(4aH)-one, 1,2,3,4,5,6-hexahydro-1,1,5,5-tetramethyl-, (2 s-cis)- (or) Isolongifolen-9-one	2.35	–	–	1627
34	43.799	Neoisolongifolene, 8-oxo-	–	–	1.50	1703
35	43.804	Isolongifolen-9-one (or) 2,4a-Methanonaphthalen-7(4aH)-one, 1,2,3,4,5,6-hexahydro-1,1,5,5-tetramethyl-, (2 s-cis)-	–	1.04	–	1703
36	44.679	(Z)6,(Z)9-Pentadecadien-1-ol	1.39	–	–	1737
37	45.115	Decane, 3-ethyl-3-methyl-	–	–	1.75	1753
38	45.885	Syn-Tricyclo[5.1.0.0(2,4)]oct-5-ene, 3,3,5,6,8,8-hexamethyl-	–	1.02	–	1782

Table 2 continued

Sl. no	RT (min)	Compound	100 bar (ppm)			RI
			40 °C	50 °C	60 °C	
39	49.431	Benzenamine, 4,5-dimethyl-2-(4-pyridylmethoxy)-	–	–	1.58	1922
40	49.446	Isobenzofuran-1(3H)-one, 3-(3-furyl)-3a,4,5,6-tetrahydro-3a,7-dimethyl-	–	0.70	–	1922
41	49.662	Pentadecanoic acid, 14-methyl-, methyl ester	–	–	9.78	1932
42	49.676	Hexadecanoic acid, methyl ester	11.56	2.35	8.88	1927
	49.681					1932
	51.287					1998
43	50.467	Tetradecane,1-iodo-	–	–	1.60	1965
44	50.567	n-Hexadecanoic acid	69.23	101.8	233.79	1969
	50.837			28.04		1980
	50.982					1986
	51.172					1994
45	51.162	(S)-(+)-6-Methyl-1-octanol	–	–	6.68	1993
	51.307	Hexadecanoic acid, ethyl ester	8.52	0.81	8.88	1999
	51.287					1998
46	53.228	11,14-Eicosadienoic acid, methyl ester	–	1.10	4.99	2083
	53.638					2100
47	53.378	trans-13-Octadecenoic acid, methyl ester	–	2.73	–	2089
48	53.658	9,12-Octadecadienoic acid, methyl ester	5.04	0.88	–	2101
	53.663					
49	53.768	6-Octadecenoic acid, methyl ester, (Z)-	–	2.07	10.85	2106
	53.783					
50	53.788	8-Octadecenoic acid, methyl ester	11.79	–	–	2106
51	54.043	Methyl stearate	1.71	1.10	–	2117
	54.353					
52	54.353	Methyl 8-methyl- nonanoate	–	–	2.69	2130
53	54.894	Cis-Vaccenic acid (or) 11- Octadecenoic acid, (Z)-	8.77	120.74	145.98	2153
	54.968					2156
	55.023					2158
	57.189					2246
54	55.249	(E)-9-Octadecenoic acid ethyl ester	2.11	1.12	4.51	2167
	55.264					2168
	55.278					2168
55	55.389	Octadecanoic acid (or) stearic acid	2.76	35.21	34.28	2173
	55.439					2175
	55.494					2177
56	55.659	Heptadecane, 9-hexyl-	–	–	7.36	2184
	63.562				5.31	2485
57	55.929	Nonadecane, 2-methyl-	–	–	12.84	2195
58	57.844	2-methyltetracosane	–	0.67	–	2271
59	61.916	Nonadecane, 2-methyl	–	0.72	–	2426
60	63.111	1,2-Benzenedicarboxylic acid, bis(6-methylheptyl) ester (or) Phthalic acid, bis(6-methylheptyl) ester	–	9.23	–	2469
61	63.827	Eicosane, 2-methyl-	–	0.61	–	2495
62	64.547	Anthracene, 9- dodecyltetradecahydro	–	–	7.02	2520
63	65.013	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	–	–	2.08	2537
64	65.843	Phthalic acid, 4-methylpent-2-yl nonyl ester	–	0.73	–	2565
	66.288			1.77		2581
	66.698			0.63		2595
65	66.858	1,2-Benzenedicarboxylic acid, dinonyl ester	–	0.94	–	2600

Table 3 Identification of neem seed (150 bar, 40, 50 and 60 °C) volatile compounds by GC–MS

Sl. no	RT (min)	Compound	150 bar (ppm)			RI
			40 °C	50 °C	60 °C	
1	16.358	Amylene hydrate	2.35	–	–	1011
2	22.706	Nonanal	–	0.74	–	1110
3	23.331	2,3-Dimethyl-4-hydroxy-2-butenic lactone	–	–	0.49	1124
4	26.547	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	–	0.91	–	1188
5	27.198	Hexan-3-yl propyl carbonate	–	–	2.53	1201
6	30.304	2,6-Octadienal, 3,7-dimethyl-, (E)-	–	0.94	–	1279
7	30.824	Nonanoic acid	8.38	2.44	1.89	1292
	30.859					1292
	30.924					1294
8	31.969	2, 4- Decadienal	1.42	0.78	1.12	1323
	31.974					1323
	31.979					1324
9	32.204	1,2,4-Trithiolane, 3,5-diethyl-	3.02	1.07		1330
	32.210					
	32.485			2.79		
	32.495				1.52	
10	32.745	1-Propene, 1,1'-thiobis-	–	2.06	–	1346
	32.755	Propanal, 2-methyl-, ethylhydrazone	–	–	1.47	1346
11	33.72	2(3H)-Furanone, dihydro-5-pentyl-	1.58	0.85	0.48	1373
	33.72					1373
	33.725					1373
12	34.125	n- Decanoic acid	1.01	–	–	1384
13	34.345	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	–	4.32	–	1390
14	34.365	(E)-3,7-Dimethylocta-2,6-dienyl ethyl carbonate	–	–	2.15	1390
15	35.831	cis-muurolo-4(14),5-diene	–	0.91	0.515	1436
	35.841					
16	35.951	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	–	2.34	1.78	1439
	35.961					1440
17	36.046	1-Propene, 3-[(1-methylethyl)thio]-	26.69	8.97	7.97	1442
	36.051					1443
	36.076					1443
18	36.361	1-Ethyl-3-vinyl-adamantane	–	0.75	–	1452
19	36.371	Germacrene D (or) 1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [S-(E,E)]-	–	–	0.49	1453
20	36.976	Spiro[4.5]dec-7-ene, 1,8-	–	2.68	1.47	1471
	36.981	dimethyl-4-(1-methylethenyl)-, [1S-(1à,4à,5à)]-				1471
21	37.507	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl	–	1.25	0.99	1487
	37.512					1488
22	37.827	Naphthalene, 1,2,3,5,6,7,8,8a-	–	5.28	2.65	1497
	37.837	octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1à,7à,8aà)]-				1497
23	37.832	à-Panasinsene	0.92	–	–	1497
24	38.297	β-Bisabolene	2.19	5.15	3.30	1513
	38.302					
	38.312					
25	39.132	Undecane, 3,8-dimethyl-	1.26	–	–	1541
26	40.22	Dodecanoic acid	1.14	–	0.70	1578
	40.243					
27	40.638	4,6-Diethyl-1,2,3,5-tetrathiolane	2.84	0.99	–	
	40.748		1.66			
28	40.873	Heptadecane, 2,6,10,14-tetramethyl-	1.95	–	–	1599
29	41.113	Diethyl Phthalate	1.29	1.18	–	1607
	41.103					

Table 3 continued

Sl. no	RT (min)	Compound	150 bar (ppm)			RI
			40 °C	50 °C	60 °C	
30	42.614	trans-Sesquisabinene hydrate	1.99	–	–	1661
31	42.829	Hexasiloxane, tetradecamethyl-	–	1.85	–	1669
32	42.889	Aromadendran	2.33	–	0.64	1671
33	43.414 43.494	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1 α ,4 α β ,8 α α)]- (or) Eudesm-7(11)-en-4-ol; Juniper camphor	49.15	–	5.81	1689 1692
34	43.529	Selina-6-en-4-ol	–	7.67	–	1693
35	43.679	4-(1,5-Dimethylhex-4-enyl)cyclohex-2-enone	–	3.23	–	1698
36	43.789 43.789 43.804	2,4a-Methanonaphthalen-7(4aH)-one, 1,2,3,4,5,6-hexahydro-1,1,5,5-tetramethyl-, (2 s-cis)-	7.19	2.61	1.60	1703 1703 1703
37	44.679 44.679 44.679	(Z)6,(Z)9-Pentadecadien-1-ol	1.87	0.69	0.59	1737 1737 1737
38	45.115	2-Bromo dodecane	1.19	–	–	1753
39	45.71 45.74	Tetradecanoic acid	1.15	–	0.56	1775 1776
40	45.875	syn-Tricyclo[5.1.0.0(2,4)]oct-5-ene, 3,3,5,6,8,8-hexamethyl-	1.82	–	–	1781
41	46.52	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(2-methyloxiranyl)-	–	1.36	–	1805
42	47.06	Heptasiloxane, hexadecamethyl-	–	1.33	–	1827
43	48.386 48.386	Phthalic acid, hex-3-yl isobutyl ester	3.47	–	0.79	1879 1879
44	49.651 49.656 49.661	Hexadecanoic acid, methyl ester	14.46	6.27	3.17	1931 1928 1932
45	50.902 50.922 51.087	n- Hexadecanoic acid	180.48	61.91	77.59	1983 1984 1990
46	51.287 51.287 51.292	Hexadecanoic acid, ethyl ester	16.38	5.67	10.66	1998 1998 1999
47	53.633	11,14-Eicosadienoic acid, methyl ester	–	2.34	–	2100
48	53.643 53.648	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (or) Linoleic acid, methyl ester	4.33	–	1.14	2100
49	53.763	6-Octadecenoic acid, methyl ester, (Z)-	–	5.06	–	2105
50	53.768 53.768	trans-13-Octadecenoic acid, methyl ester	11.05	–	2.84	2106
51	54.283	Hexasiloxane, tetradecamethyl-	–	1.34	–	2127
52	54.333 54.348	Methyl stearate	–	1.32	0.53	2129
53	54.343	Hexadecanoic acid, 15-methyl-, methyl ester	1.68	–	–	2130
54	54.908 54.913 55.009	Cis-Vaccenic acid (or) 11-Octadecenoic acid, (Z)-	62.30	27.09	26.57	2153 2153 2157
55	55.239 55.244 55.254	9-Octadecenoic acid ethyl ester	1.84	0.68	1.76	2167 2167 2167
56	55.389 55.394 55.459	Octadecanoic acid (or) Stearic acid	11.62	5.37	5.41	2173 2173 2176

Table 4 Identification of neem seed (200 bar, 40, 50 and 60 °C) volatile compounds by GC–MS

Sl. no	RT of sample	Compound	200 bar (ppm)			KI
			40	50	60	
1	4.023	Trichloromethane	–	–	4.73	744
	4.879					778
2	4.078	Methane, oxybis[dichloro-	–	6.63	–	747
3	4.824	Desmethyldeprenyl	–	0.33	–	776
4	5.234	Difluorophosphoric acid	–	0.21	–	790
5	16.303	Butanoic acid	–	–	2.15	1010
6	21.795	Pyrazine, tetramethyl-	4.45	–	–	1094
7	23.056	Acetic acid, 2-(thiocarboxy)hydrazide, O-methyl ester	3.79	–	–	1118
8	23.326	4-Hexen-3-one, 4-methyl	3.80	–	–	1124
9	26.192	3-Cyclohexen-1-ol, 4-methyl-1-	7.74	–	5.26	1182
	26.202	(1-methylethyl)-, (R)-				1182
10	26.992	Octanoic acid	13.54	–	12.72	1197
	27.143					1199
11	30.274	2,6-Octadienal, 3,7-dimethyl-, (E)-	6.55	–	–	1278
12	30.759	Nonanoic acid	9.20	–	13.47	1290
	30.864					1292
13	31.935	2,4-Decadienal	13.99	–	3.37	1322
	31.944					1322
14	32.16	1,2,4-Trithiolane, 3,5-diethyl-	6.39	–	–	1329
	32.445		16.15			1337
	32.705		15.60			1344
15	34.055	n-Decanoic acid	–	–	1.50	1382
16	35.911	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)	11.29	–	–	1438
17	36.931	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- or α -Curcumene	7.13	–	–	1470
18	37.787	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1 α ,7 α ,8 α)]-	15.42	–	–	1496
19	38.257	β -Bisabolene	23.77	–	–	1511
20	38.622	6-Fluoro-2-trifluoromethylbenzoic acid, 2,3-dichlorophenyl ester	–	–	5.44	1524
21	39.087	Tetradecane, 1-iodo-	–	–	2.40	1540
22	40.593	4,6-Diethyl-1,2,3,5-tetrathiolane	5.33	–	–	1590
23	42.834	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1 α ,4 α ,8 α)]-	–	–	141.82	1669
24	42.859	1H-Cycloprop[<i>e</i>]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1aR-(1 α ,4 β ,4 α ,7 α ,7 α ,7 β)]- or viridiflorol	70.59	–	–	1670
25	43.739	Neoisolongifolene, 8-oxo-	–	–	2.32	1701
26	45.065	1-Iodoundecane	–	–	2.62	1751
27	45.82	Benzene, 1-[1,1-dimethylethyl]-4-[2-propenyloxy]-	–	–	1.86	1779
28	47.03	Heptasiloxane, hexadecamethyl-	–	–	10.25	1826
29	47.085	Isopropyl myristate	5.09	–	–	1828
30	49.601	Pentadecanoic acid, 14-methyl-,	7.54	–	14.70	1929
	49.602	methyl ester				1929
31	50.677	Phthalic acid, butyl tridecyl ester	40.16	–	–	1974
32	50.752	n-Hexadecanoic acid	300.13	30.67	214.16	1977
	50.812					1979
	50.892					1982
33	51.227	Heptanoic acid, 2-ethyl-	–	–	3.11	1996
34	53.573	2-Tetradecyne	3.74	–	3.21	2097
	53.588					2098
35	53.708	9-Dodecenoic acid, methyl ester, (E)-	–	–	9.55	2103
36	54.248	Hexasiloxane, tetradecamethyl-	–	–	11.40	2126
37	54.628	9-Decen-1-ol	–	–	5.38	2142

Table 4 continued

Sl. no	RT of sample	Compound	200 bar (ppm)			KI
			40	50	60	
38	54.854	cis-Vaccenic acid or 11-	–	25.40	–	2151
	54.854	Octadecenoic acid, (Z)-				2151
39	55.079	Spiro[2.5]octane	–	0.29	–	2160
40	55.239	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	–	–	20.43	2167
41	55.324	Octadecanoic acid	47.91	9.50	–	2170
	55.329					2171
42	57.384	Hexasiloxane, tetradecamethyl-	–	–	7.91	2253
43	58.18	Naphthalene, decahydro-1-pentadecyl-	–	0.13	–	2285
44	60.346	Triphenyl phosphate	–	0.13	–	2368
45	63.607	Oxirane, hexadecyl	20.18	–	–	2487
46	65.663	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	4.54	–	–	
47	67.324	Dodecane, 1-cyclopentyl-4-(3-cyclopentylpropyl)-	3.26	–	–	2616

Table 5 Identification of neem seed (Hydro-distillation) volatile compounds by GC–MS

Sl.no	RT (min)	Compound	RI	(ppm)
1	7.135	1,3-Propanedithiol	844	3.46
2	23.531	Allyl mercaptan	1128	2.60
3	26.787	1-Dodecene	1193	1.35
4	28.353	4,6-Dimethyl-[1,2,3]trithiane	1231	4.58
5	31.344	1,3-Propanedithiol	1305	1.51
6	32.275	1,2,4-Trithiolane, 3,5-diethyl-	1332	58.89
7	32.59	1,2,4-Trithiolane, 3,5-diethyl-	1341	77.57
8	33.545	1,2,4-Trithiolane, 3,5-diethyl-	1368	0.92
9	34.446	1-Hexadecanol	1393	9.64
10	34.566	1,2,4-Trithiolane, 3,5-diethyl-	1396	2.34
11	35.416	1H-3a,7-Methanoazulene, octahydro-3,8,8-trimethyl-6-methylene-, [3R-(3a,3aa,7a,8aa)]-	1423	0.75
12	35.951	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	1439	3.50
13	36.366	γ -Murolene	1452	1.19
14	36.977	Spiro[4.5]dec-7-ene, 1,8-dimethyl-4-(1-methylethenyl)-, [1S-(1 α ,4 β ,5 α)]-	1471	2.51
15	37.197	5,6-Dihydro-2,4,6-triethyl-4H-1,3,5-dithiazine	1478	4.41
16	37.537	Cycloisolongifolene	1488	1.01
17	37.837	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1 α ,7 β ,8a α)]-	1497	4.88
18	38.307	α -Bisabolene	1513	3.84
19	38.712	Phenol, 2,4-bis(1,1-dimethylethyl)-	1527	9.60
22	40.088	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-	1573	0.74
23	40.703	1-Hexadecanol	1593	16.89
24	42.919	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1 α ,4a β ,8a α)]-	1672	4.94
25	46.22	E-15-Heptadecenal	1794	15.23
26	47.056	1,2,4-Trithiolane, 3,5-diethyl-	1827	13.75
27	47.646	1,2,4-Trithiolane, 3,5-diethyl-	1850	14.72
28	47.821	1,2,4-Trithiolane, 3,5-diethyl-	1857	6.15
29	50.162	Oxacycloheptadec-8-en-2-one, (8Z)	1952	1.06
30	51.192	Cycloeicosane	1995	13.36
31	52.563	4,6-Diethyl-1,2,3,5-tetrathiolane	2054	1.52
32	55.734	n-Tetracosanol-1	2187	8.54
33	59.911	n-Tetracosanol-1	2351	3.20
34	63.782	9-Hexacosene	2493	1.13

comparison with other SCF parameters and hydro-distillation process.

Conclusion

Recently, supercritical fluid carbon dioxide is used for the extraction process of bioactive compounds to get near natural forms without any artifact formation. Neem seed powder was subjected to SCF at different pressure and temperatures. Extracts were partitioned to separate volatiles and were analyzed by GC–MS along with the volatiles extracted by conventional method. There is a significant effect of pressure and temperature on isolation of number of volatile compounds as well as retention of biological active compounds by SCF. Extraction at 100 bar, 40 °C showed forty volatile compounds corresponds to 92.39% of volatiles with the major of bioactive compounds such as Terpinen-4-ol, 1,2,4-Trithiolane, 3,5-diethyl, allyl isopropylsulphide, Cycloisolongifolene, α -Bisabolene, (–)- α -Panasinsen, Isocaryophyllene, trans-Sesquisabinene hydrate, 1-Naphthalenol.

Acknowledgements The authors are thankful to the Director of CSIR-CFTRI for providing facilities and encouragement. The first author Swapna sonale R. acknowledges the Indian Council of Medical Research, India for providing Senior Research Fellowship.

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