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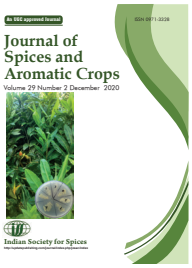

Volatile metabolites of endophytic *Klebsiella aerogenes* from *Zingiber zerumbet* rhizome and its antagonistic effect on soft rot causative *Pythium myriotylum*

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Abstract

Rhizomes of *Zingiber zerumbet* collected from their natural habitat and reported earlier to have high zerumbone content were selected for isolation of endophytes. Biochemical and molecular characterization using 16S rRNA sequencing of the endophytes identified the isolates as belonging to genus *Klebsiella*, *Pantoea* and *Enterobacter*. Isolate designated *ZzKSD*8 identified as *K. aerogenes* yielded maximal antagonistic activities against *P. myriotylum* determined as 83.5% ± 0.77. Volatile metabolites produced by *ZzKSD*8 caused absolute impairment of *P. myriotylum* hyphal growth compared to control (23.9 ± 0.37 cm). Volatile metabolites were extracted from 48 hour grown stationary phase *ZzKSD*8 cultures using absolute methanol, ethyl acetate and ethanol (60% v/v). GC-MS metabolite profiling detected alkanes and fatty acid methyl esters as the predominant constituents in the solvent extracts. Major constituents included methyl palmitate (31.37%), methyl stearate (18.57%) and cyclopropaneoctanoic acid, 2-hexyl-, methyl ester (17.05%) in methanol extract; alkanes like tetratetracontane (13.18%) and 2-methyloctacosane (12.10%) in ethyl acetate extracts and 4,22-sigmastadiene-3-one as major metabolite (22.51%) and stigmast-5-en-3-ol, (3.beta) (17.40%) in ethanol extract. Identified metabolites reported to modulate defense strategies in plants against phytopathogens makes *ZzKSD*8 a potential candidate for development of biological alternatives to control soil-borne soft-rot disease.

Keywords: antagonistic, endophyte, soft-rot, volatile organic compounds

Introduction

Compared to foliar pathogens for which resistance is often encoded in plant genome, finding resistance factors against soil-borne pathogens is challenging. Roots and rhizomes with their nutrient reserves tend to attract microbes to rhizosphere (Shubin *et al*. 2014; Santoyo *et al*. 2016). As a consequence there exists increased competition and dynamics

in the soil habitat (Raaijmakers *et al*. 2009) making it difficult to manage soil-borne pathogens. Rhizosphere dynamics is regulated by the metabolites produced from below-ground tissues like roots and rhizomes that create specific niches and thereby foster beneficial microbial associations (Shubin *et al*. 2014; Jia *et al*. 2016). Some of these microbes form mutualistic associations as endophytes benefiting growth and defense of host plants (Mendes *et al*. 2013; Adedeji & Babalola 2020). Host genotype is known to significantly influence endophytic colonization (Overbeek & Elsas 2008; Shubin *et al*. 2014) as in wheat cultivars wherein differences in microbial composition were observed based on varying resistance levels (El Arab *et al*. 2001). Thus endophytic microbes isolated from resistant taxon root/ rhizome with allelopathic potential can serve as candidates for development of biocontrol agents for plant protection.

Endemic plants with ethnomedicinal properties harbors diverse endophytic microbes making them ideal source for prospecting candidate endophytes with biocontrol potential (Strobel & Daisy 2003). Tropical medicinal plant, *Zingiber zerumbet* is thus ideal for prospecting endophytes due to its wide medicinal properties (Chien *et al*. 2008) and robust resistance to soft-rot causative *Pythium* species (Nair & Thomas 2013), a necrotrophic pathogen causing extensive economic loss due to its wide host range. Preliminary study on prospecting *Z. zerumbet* endophytes led to the isolation of fungal isolates of *Fusarium* genus with antagonistic activity to *Pythium* spp. (Keerthi *et al*. 2016). Compared to fungal endophytes, bacterial endophytes with plant growth promoting (PGP) traits (Gaiero *et al*. 2013) have been identified to hold greater potential for development of biocontrol agents and as soil amendments (Ryan *et al*. 2007; Chaparro *et al*. 2012). This is owing to higher efficacy and survival of bacterial endophytes with PGP traits in the competitive soil environment due to production of allelochemicals with antagonistic activity (Chaparro *et al*. 2012).

The principal secondary metabolite in *Z. zerumbet* namely zerumbone which contributes to antagonistic activity towards *Pythium* species (Keerthi *et al*. 2014) has been reported to vary based on geographical location with highest zerumbone content observed in accessions from Kasaragod District of Kerala (Baby *et al*. 2009). Such habitat induced variations in metabolite content can therefore influence the rhizosphere microbial composition (Aleklett *et al*. 2015). Against this backdrop, present study was undertaken to (i) isolate and characterize antagonistic bacterial isolates from the rhizome of *Z. zerumbet* accessions collected from Kasaragod and (ii) characterize the metabolites produced by selected isolate(s).

Materials and methods

Sampling and microbial isolation

Rhizomes of healthy *Z. zerumbet* plants were collected from Kasaragod District, Kerala (Latitude: 11.7381° N, Longitude: 76.0740° E). Collected rhizomes were immediately sealed in plastic bags and transported under ice to laboratory. Soil and debris sticking to rhizomes were removed by washing under running tap water for 30 minutes. Washed rhizomes were successively treated with ethanol (70% v/v) for 1 min; rinsed with sterile water; treated with sterilization solution (NaOCl containing two drops of Tween 20; 20% v/v) for 5 min; rinsed with sterile water; treated with mercuric chloride (0.1% w/v) for 8 min followed by three washes with sterile water. Aliquot of last wash water (1 ml) was plated on LB medium to evaluate the effectiveness of surface sterilization procedure. Treated rhizome was cut into pieces and ground using sterile mortar and pestle (Zhang *et al*. 2018) and suspended in Luria Bertani (LB) broth. The obtained rhizome homogenate was plated (20 µl) on freshly prepared LB agar and incubated at 25°C for 24-72 hour. Endophytic isolates obtained were

characterized and stored as glycerol stocks at -80°C.

Morphological and biochemical characterization of rhizome endophytes

For cultural and morphological characterization, isolates were grown overnight in LB plates and LB broth at 25 ± 3°C. Cultures were observed to determine colony surface, margin and opacity. Further biochemical characterization like gram staining, motility, endospore staining, catalase activity, methyl red test, gelatin hydrolysis and citrate utilization ability of all the bacterial isolates were determined for taxonomic identification.

Molecular and phylogenetic characterization

Genomic DNA was extracted from overnight grown cultures following CTAB protocol (William *et al*. 2012). PCR amplification was carried out using 16rDNA universal primers, 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SRev (5'-GGTTACCTTGTTACGACTT-3'). PCR reaction mixture (20 µl) contained 20 ng DNA, 10 pmol of each primer, 10 mM each dNTP, 10X PCR reaction buffer with 1.5 mM MgCl2 and 0.6U Taq DNA polymerase. Thermo-cycling was carried out in S1000 Thermal cycler (Bio Rad, USA) and consisted of initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were electrophoretically examined in 1.2% agarose gel, excised from the gel and purified using Wizard SV gel and PCR Clean-up System (Promega, WI, USA) following manufacturer's instruction prior to sequencing. Sequences were subjected to homology searches using BLASTN algorithm (http://www.ncbi.nlm.nih.gov/) in NCBI database and aligned using CLUSTAL W (Thompson *et al*. 1994) with homologous sequences retrieved from NCBI database. Phylogenetic relationship of endophytic isolates was inferred from the maximum likelihood (ML) (Felsenstein 1981) tree generated using MEGA 5 software (Kumar *et al*., 2018). Bootstrap analysis was carried out with 1000 datasets to check the robustness of the constructed phylogenetic tree.

Dual culture assay of endophytic isolates

Dual culture assay was conducted to evaluate the antagonistic effect of endophytic bacterial isolates against soft-rot causative *Pythium myriotylum* strain (RGCBN14), which was obtained from Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, Kerala. The isolates were streaked on one side of the PDA plate (pH– 6.5) and the *Pythium* hyphal disc (5 mm) was placed at opposite side. The plates were incubated at 25 ± 3°C for 7 days and antagonistic activity determined as percentage of inhibition (PoI) was determined as: PoI = [(R1-R2)/R2] x 100 (Keerthi *et al*. 2016).

Evaluation of VOCs produced by KSD8 isolate against P. myriotylum

Effect of VOC produced by *ZzKSD*8 was determined by double plate technique. For the same, bacterial lawn was prepared on LB agar medium at 25 ± 3°C. The lid of the petri-plate was replaced with lid of another plate that was inoculated with *P. myriotylum* (RGCBN14) hyphal disc (5 mm) on PDA medium. The plates were sealed with parafilm to prevent leak of bacterial volatile compounds and incubated at 28 ± 3°C in dark for five days. The diameter of *P. myriotylum* hyphal growth was measured after the incubation period and PoI was calculated. Assays were performed three times with three replicates.

Metabolite profile of KSD8 isolate by GC-MS

Metabolite(s) produced by endophytic bacterial isolate, *ZzKSD*8 that exhibited significant antagonistic activity was evaluated. For the same, bacterial growth curve was determined for a period of 2 hours to 8 days. Metabolites were subsequently extracted using three solvents namely, absolute methanol, ethyl acetate and ethanol (60% v/v) from the stationary phase

cultures. For solvent extraction, cultures were centrifuged at 10,000 rpm for 5 minutes to sediment the bacterial cells. Pelleted cells were suspended in pure solvent (1 ml/ 5 ml culture volume), shaken overnight and the suspension was sonicated the next day. The homogenate was centrifuged at 10,000 rpm for 15 min at 10°C and the supernatant was filtered (0.22 µm filter) and subjected to GC-MS analysis.

GC-MS analysis was done using high resolution Agilent GC 7890A (injector temperature 250°C) coupled to Agilent 5975C mass detector in the split mode of 50:1. Separation were carried out in a DB 5 MS column with dimensions 30 m × 0.25 mm × 0.25 µm. Carrier gas used was helium with a flow rate of 1.0 mL min^{-1}. Initial temperature of the oven was 40°C with an increase of 5°C min^{-1} to 280°C. Peaks obtained were identified by matching the mass spectra in Wiley and National Institute of Standards and Technology (NIST) Mass Spectral Library.

Results and discussion

Endophytic bacterial isolates from Z. zerumbet *rhizome*

Z. zerumbet rhizomes were collected from their natural habitat in Kasaragod, reported earlier to have the highest zerumbone content (Baby *et al*. 2009). Our earlier endophyte isolation procedure from *Z. zerumbet* had used sterilized rhizome slices (Keerthi *et al*. 2016) and thus differs present procedure wherein ground rhizome was used as it ensures sampling from the internal plant tissue. Morphologically distinct bacterial colonies were selected and characterized for color, shape, elevation, texture and margins. The morphological features of the ten isolates obtained were tabulated (Table 1). Biochemical characterization of isolates revealed all to be gram negative except isolate *ZzKSD*1 which was also a spore producer; rod-shaped; motile except three isolates namely, *ZzKSD*2, *ZzKSD*5 and *ZzKSD*8 and all isolates were also catalase positive. Isolates also yielded positive results for methyl red test and fermentation of sugars, glucose, fructose and sucrose. Isolate *ZzKSD*8 was positive for indole test and citrate utilization. Gelatin liquefaction was positive only for *ZzsKSD*9 while all isolates were negative for sulphide test (Fig 1).

	Gram staining	Shape	Motility	Spore staining	Catalase	Metyl red test	Sulphide test	Indole test	Gelatin liquifaction test	Citrate utilization test	Sugar fermentation		
											Glu	Fru	Suc
*ZzKSD*1	+	Rod	+	+	+	+++	-	-	-	-	+	+	+
ZzKSD2	-	Rod	-	-	+	++	-	-	-	-	+	+	+
ZzKSD3	-	Rod	+	-	+	++	-	-	-	-	+	+	+
ZzKSD4	-	Rod	+	-	+	++	-	-	-	-	+	+	+
ZzKSD5	-	Rod	-	-	+	++	-	-	-	+	+	+	+
ZzKSD6	-	Rod	+	-	+	+	-	-	-	-	+	+	+
ZzKSD7	-	Rod	+	-	+	+	-	-	-	-	+	+	+
ZzKSD8	-	Rod	-	-	+	++	-	+	-	+	+	+	+
ZzKSD9	-	Rod	+	-	+	+++	-	-	+	-	+	+	+
ZzKSD10	-	Rod	+	-	+	+	-	-	-	-	+	+	+

Fig. 1. Morphological and biochemical characteristics of bacterial endophytes isolated from *Z. zerumbet* rhizomes.

PCR amplification using 16S rRNA yielded amplicons of ~1.4 kb which were eluted, sequenced and subjected to homology searches using BLASTn algorithm at NCBI (Table 1). Following multiple sequence alignment using CLUSTALX, phylogenetic analysis was carried out and the ML tree clustered the sequences with homologs which was supported by high bootstrap values (Fig. 2). The sequences have been deposited in the GenBank database under the accession numbers: MW369495- MW369500. At sequence identity threshold >99%, homology searches revealed isolates *ZzKSD*2, *ZzKSD*5 and *ZzKSD*8 to show similarity to *Klebsiella* strains (Table 1; Fig. 2). Isolate *ZzKSD*1 showed homology to *Bacillus* spp. (Accession no. KC466190.1; 100% identity); *ZzKSD*3, *ZzKSD*4 and *ZzKSD*9 were homologous to *Pantoea* spp. (Accession no. MK612131.1; 99.8% identity); *ZzKSD*6, ZzKSD7 and *ZzKSD*10 to *Enterobacter tabaci* strain (Accession no. MK641315.1; 100% identity). Members of Enterobacteriaceae family (Gammaproteobacteria) that includes species

Table 1. Morphological and molecular characterization of endophytic isolates from *Z. zerumbet* rhizome

Isolate	Morphological and culture characteristics	16S rRNA gene sequencing and homology (GenBank Accession Number)	Sequence identity (%)
*KSD*1	Pale cream color, small size, smooth surface, opaque, entire margin, elevation is flat and in circular form	*Bacillus* spp. (KC466190.1)	100.00
*KSD*2	White color, small colonies, surface is smooth, entire margin, translucent, elevation is flat and in circular form	*Klebsiella* spp. (U32868.1)	99.65
*KSD*3	Yellow color, small colonies, smooth surface, entire margin, translucent, convex elevation and in circular form	*Pantoea* spp. (MK612131.1)	99.78
*KSD*4			
*KSD*5	Pale white, small colonies, smooth surface, opaque, entire margin, convex elevation and in circular form	*Klebsiella aerogenes* (MF682950.1)	100.00
*KSD*8			
*KSD*9	Pale white, medium size colony, smooth surface, entire margin, flat and in circular form	*Pantoea dispersa* (MH542280.1)	99.86
*KSD*6	White color, small size, smooth surface, translucent structure, entire margin, elevation is convex and in circular form	*Enterobacter tabaci* (MK641315.1)	100.00
*KSD*7			
*KSD*10			

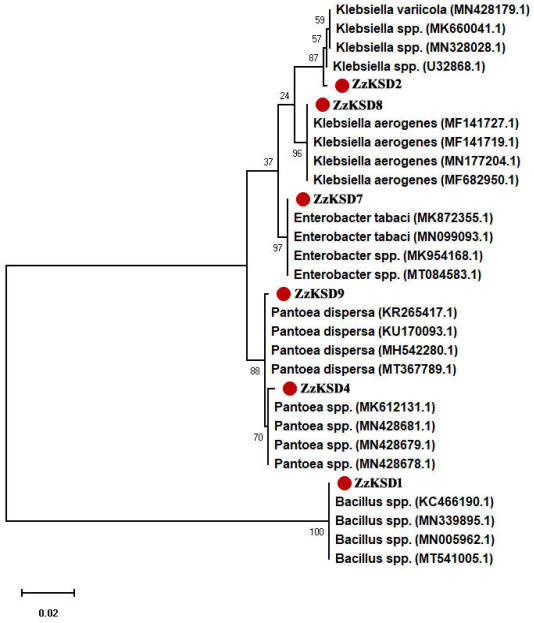

Fig. 2. Maximum likelihood tree based on 16S rRNA gene sequences showing the identity of endophytes from *Z. zerumbet* rhizome with homologous sequences identified following NCBI searches. Genbank accession number of microbial sequences used for phylogenetic analysis are given at the end of each node and the *Z. zerumbet* endophytes are indicated in figure by prefixing with filled circles. Numbers at nodes indicate bootstrap values.

of genus *Klebsiella*, *Pantoea* and *Enterobacter* constitute important rhizosphere colonizers (Torres *et al*. 2008) and include diazotrophs with N_2 fixing ability (Banik *et al*. 2016). Despite being taxonomically related to nosocomial isolates causing human disease, *Klebsiella* spp. have been identified as endophytes from various plants (Martinez *et al*. 2003; Mendes *et al*. 2013) like maize wherein they have been shown to enhance plant growth and were distinct from clinical isolates lacking virulence factors (Dong *et al*. 2003). Furthermore, diazotrophic *Klebsiella* spp. are of agricultural interest and constitute excellent model organisms for study of nitrogen fixation (Hoover 2000). Epiphytic and endophytic *Pantoea* spp. and *Enterobacter* spp. exhibit plant growth promoting traits with excellent biocontrol potential (Mishra *et al*. 2011; Macedo-Raygoza *et al*. 2019). *Bacillus* spp. are ubiquitous endophytes in diverse plants and have been reported as potential candidate BCAs (Nigris *et al*. 2018).

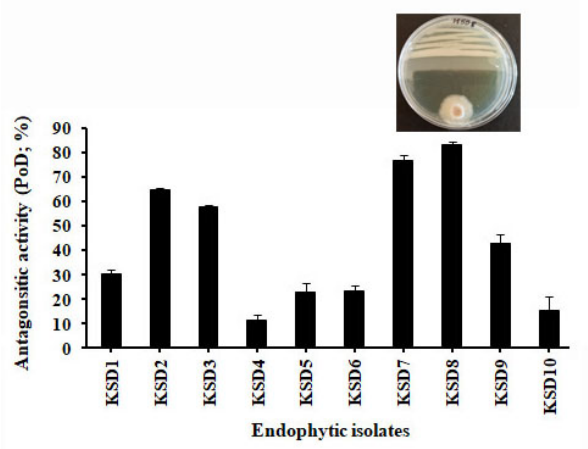

Fig. 3. Antagonsitic activity of endophytes from *Z. zerumbet* rhizome against *P. myriotylum* as determined by dual culture assay. Each value is the mean of four replicates (n = 4) with standard error shown as error bars. Inset shows the inhibitory effect of *ZzKSD*8 towards *P. myriotylum*.

Identification of isolates with antagonistic potential

Amongst the 10 *Z. zerumbet* endophytic isolates evaluated by dual culture or confrontation assays for antagonistic activities against *P. myriotylum*, *ZzKSD*8 yielded highest percentage of inhibition (83.5 ± 0.77) (Fig. 3) and thereby constitute potential candidate for soft-rot disease control. With *Pythium* species gaining entry through rhizomes, the endophyte characterized from rhizome constitute ideal candidates for developing BCAs to prevent soil-borne soft-rot disease. Root associated endophytes have been reported to mediate antagonistic activities against phytopathogens in various plants by *de novo* synthesis of metabolites that interfere with pathogenicity factors (Jia *et al*. 2016). These associations have been investigated in various plants such as maize, wheat, oat, barley, peas, canola, soy, potatoes, tomatoes, lentils and cucumber (Ryan *et al*. 2007). With *ZzKSD*8 showing homology to *K. aerogenes*, identification of volatile metabolites will have implications in both phytopathology and human health.

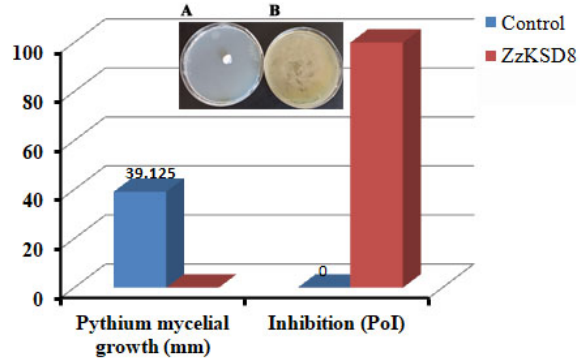

Fig. 4. Inhibitory potential of volatile organic compounds (VOCs) of *ZzKSD*8 towards *P. myriotylum* growth. Double plating technique was used to determine the inhibitory effect on (A) *P. myriotylum* mycelial growth (radius in mm) by (B) volatiles of *ZzKSD*8 spread-plated on bottom plate as shown in inset. The graph in the figure shows the mycelial growth (mm) and PoI as determined from the experiments

Volatile secondary metabolites of ZzKSD8 isolate

Volatile secondary metabolites constitute a major class of organic compounds produced by soil bacteria besides the soluble metabolites (Tyc *et al*. 2017). Biocontrol activity of many biocontrol endophytes has been attributed to production of volatile metabolites. Present experiments following double plate technique revealed that the volatile metabolites produced by *ZzKSD*8 caused impairment in *P. myriotylum* hyphal growth compared to control (39.12± 0.37 mm) experiments yielding 100% PoI (Fig. 4). Double plate experiments thus reveal absolute antagonistic activity towards *P. myriotylum* (Fig. 4 inset A) by *ZzKSD*8 (Fig. 4 inset B). Secondary metabolites are known to accumulate during stationary growth phase when there is nutrient depletion (Ruiz *et al*. 2010). Growth curve analysis of *ZzKSD*8 isolate was carried out to determine the time stationary phase is attained. Experiments revealed the stationary phase of *ZzKSD*8 isolate at 48 hour and subsequently volatile metabolite extraction was carried out from 48 hour old cultures using absolute methanol, ethyl acetate and ethanol (60% v/v) for GC-MS analysis. Predominant metabolites identified in the solvent extracts [cut-off Area (%) ≥ 10] were fatty acid methyl esters followed by sterols and alkanes. Fatty acid methyl esters like methyl palmitate (31.37%), methyl stearate (18.57%) and cyclopropaneoctanoic acid, 2-hexyl-, methyl ester (17.05%) were detected in the methanol extract; sterols like 4,22-sigmastadiene-3-one (22.51%) and stigmast-5-en-3-ol, (3.beta) (17.40%) were detected in ethanol extract and alkanes like tetratetracontane (13.18%) and 2-methyloctacosane (12.10%) were the major constituents in ethyl acetate extracts (Table 2). Fatty acid methyl esters like methyl palmitate and methyl stearate have been reported to possess nematicidal activity (Lu *et al*. 2020). Alkanes like tetratetracontane have been previously identified as volatile metabolites produced by endophytes with plant growth promoting and antimicrobial properties (Jishma *et al*. 2017). Metabolites produced by endophytes are known to modulate chemical defense strategies employed by the host plant against the invading plant pathogens. Isolate *ZzKSD*8 with antagonistic activity towards *P. myriotylum* is a putative candidate for development of biological alternatives for control of soil-borne soft-rot disease.

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Table 2. Volatile secondary metabolites identified by GC-MS analysis of absolute methanol, ethyl acetate and ethanol (60% v/v) extracts of *ZzKSD*8 isolate

Compound name	Area %	Solvent extract
Fatty acid acyl ester		
Methyl palmitate (28.401)	31.37	Methanol
Methyl stearate (32.220)	18.57	
Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester (30.037)	17.05	
10-Octadecenoic acid, methyl ester (31.876)	7.57	
9-Octadecenoic acid (Z)-, methyl ester (33.786)	1.79	
Methyl decanoate (30.392)	1.43	
Butanoic acid, 3-methyl-, 2-furanylmethyl ester (30.508)	0.72	
Ethyl stearate (24.165)	3.91	Ethanol
Ethyl palmitate (19.614)	1.99	
Docosanoic acid, ethyl ester (19.675)	0.86	
l-Norvaline, N-(2-methoxyethoxycarbonyl)-, tetradecyl ester (22.726)	0.58	
Sterol		
4,22-S8gmastadiene-3-one (34.932)	22.51	Ethanol
Stigmasta-5-en-3-ol, (3.beta.) (31.792)	17.40	
Stigmasta-5,22-dien-3-ol (26.346)	11.82	
Stigmasta-4-en-3-one (37.287)	9.29	
Cholest-4-en-3-one (33.706)	8.90	
Stigmasta-5,22-dien-3-ol, (3.beta.,22e) (26.567)	0.94	
Stigmasterol (26.017)	0.79	
Cholestan-3-ol, (3.beta.,5.alpha.) (32.400)	0.70	
Cholesta-3,5-dien-7-one (35.825)	0.65	
Alkanes		
2-methyloctacosane (41.477)	14.34	Ethyl acetate
Tetratetracontane (42.904)	13.18	
3,3-Dimethyl hexane (13.125)	2.26	
Hexane, 3,3-dimethyl (18.618)	1.28	
Hexane, 2,3,4-trimethyl (23.560)	0.93	
Decane, 2,3,8-trimethyl (29.800)	0.59	Methanol
Terpenoid		
Lup-20(29)-en-3-yl acetate (47.090)	3.61	Methanol
9,19-Cyclolanost-24-en-3-ol, (3.beta.) (35.424)	5.35	Ethanol
Cucurbitacin b, 23,24-dihydro-25-deacetoxy (37.500)	1.14	
Cycloeucalenyl acetate (34.538)	1.04	

Compound name	Area %	Solvent extract
Alkenes		
(Trans)-2-Nonadecene (29.670)	2.60	Methanol
Fatty alcohols		
1-Eicosanol (32.008)	1.66	Methanol
1-Tetradecanol (25.701)	1.61	
n-Nonadecanol-1 (33.372)	0.98	
Alkynes		
1-Nonyne (37.767)	1.52	Ethyl acetate
Others		
1,2-Benzenedicarboxylic acid (38.943)	5.15	Methanol
3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5- tris(trimethylsiloxy)tetrasiloxane (17.979)	1.45	
2-Decylthirane (28.044)	1.40	
Oxalic acid, allyltetradecyl ester (38.467)	0.71	
Cyclohexane, 1,1′-(1,3-propanediyl)bis (31.210)	0.67	
Tetraddecahydrobenzo[a] cyclodecene (38.835)	0.57	
N(1)-Cycloheyl-n(1)-[(2′-oxo-6′-chloro-2h-benzopyran-3′-yl)carbonyl]-n(2)-cyclohexylurea (39.175)	0.53	
2-Tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxybenzyl) phenol (44.168)	5.42	Ethanol
3,5-Decadien-7-in, 6-tert.butyl-2,2,9,9-tetramethyl (32.461)	2.42	
1,3,5-Trisilacyclohexane (29.519)	1.06	
4,4′-((p-Phenylene) diisopropylidene)diphenol (40.850)	3.54	Ethyl acetate
2-Propyldecan-1-ol (27.983)	3.33	
Heptcosane (47.630)	3.28	
Tridecyl acetate (33.794)	2.97	
Sulfurous acid, dodecyl 2-propyl ester (36.877)	2.62	
Chlorocarbonylsulfenyl chloride (44.525)	2.07	
Tetradecane, 2,6,10-trimethyl (38.978)	1.75	
Cytidine, 2′-deoxy (37.135)	1.62	
Beta.-d-glucopyranose, 1,6-anhydro (44.967)	1.06	
Decane, 3,4-dimethyl (32.742)	1.03	
Ethyl iso-allocholate (47.398)	0.97	
2,4-Dimethylfuran (38.125)	0.90	
3-Pyridinol-1-oxide (39.065)	0.90	
2,4-Dipropyl-5-ethyl-1,3-dioxane (40.975)	0.83	

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