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Natural products of *Nothophoma multilocularis* sp. nov. an endophyte of the medicinal plant *Rhazya stricta*

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Abstract
In the present study, we isolated endophytic fungi from the medicinal plant *Rhazya stricta* from Saudi Arabia. Twenty-eight fungal isolates representing five species were isolated from 21 leaves (10 young and 11 old) of *R. stricta*. These fungi include two species of *Alternaria*, *Aspergillus* sp., *Nothophoma* sp. and one species producing sterile mycelia. Based on morphology and phylogenetic analyses of LSU rDNA, we describe *Nothophoma* (E-2-5) as a new species to science. *Nothophoma multilocularis* is characterized by its large multiloculate pycnidia and its larger conidial dimensions than the six described *Nothophoma* species. A table (Table 3) comparing the morphology and the host of the seven *Nothophoma* species is provided, along with a key for their identification. The culture filtrates of the isolated endophytic fungi were extracted using ethyl acetate and were tested against pathogenic microbes. Fifty-five bioactive chemical compounds were identified from the crude extracts of *Nothophoma multilocularis* using GC-MS. Ten major bioactive compounds were recorded namely: Di-n-octyl phthalate representing 53.98 % of the crude extract, 2-Allyl-3,4-dimethoxybenzaldehyde (10.26 %), Maltol (9.45 %), Cetene (2.73 %), 1-Tetradecene (2.07 %), E-15-Heptadecenal (2.06 %), 2,5-Cyclohexadien-1-one (1.88 %), 1-Octadecene (1.36 %), Diethyldithiophosphinic acid (1.17 %) and Phenol, 2,4-di-t-butyl-6-nitrophenol (1.07 %). These compounds showed strong antimicrobial activity in combination.

Key words – Antibacterial – antifungal – Didymellaceae – molecular phylogeny

Introduction

Rhazya stricta Decne is a native, perennial, poisonous, evergreen dwarf shrub plant in India and the Middle East including Saudi Arabia (Täckholm 1974, Boulos 1995). *Rhazya stricta* is an important traditional medicinal species used to cure various diseases in South Asia and the Middle East. Leaf extracts from *R. stricta* have been used in folkloric medicine for the treatment of fever, sore throat, rheumatism, diabetes, inflammatory conditions and syphilis (El-Ghonemy 1993, Adam 1998). *Rhazya stricta* is a member of Apocynaceae that includes about 1300 species in 300 genera and most of which have medicinal and economic values (Täckholm 1974, Boulos 1995). Leaf extracts contain alkaloids, glycosides, flavonoids, tannins and triterpenes (Baeshen et al. 2014,

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2015). Some of its alkaloids have also been reported to have anticancerous properties (Gilani et al. 2007). Over 100 alkaloids have been isolated, characterized and identified from *R. stricta* leaves (e.g. Andersen et al. 1987, Atta-ur-Rahman & Khanum 1985, 1987), stems (Atta-ur-Rahman et al. 1996), roots (Atta-ur-Rahman et al. 1996) and legumes (Atta-ur-Rahman & Malik 1985, 1987), as well as from mixtures of aerial parts (Sultana et al. 2005). Seeds of *R. stricta* are a good source of unsaturated oil which can be used as a feedstock for biodiesel production (Nehdi et al. 2014).

It has been postulated in many studies that the internal fungi or endophytes may be responsible in producing some of the secondary metabolites found in plants (Li et al. 2005, Nisa et al. 2015). Endophytes are ecological group; most of them belong to Ascomycota and live inside healthy living tissues of every plant on earth (Guo et al. 2001, Wang et al. 2005, Hyde & Soytong 2008). Fungi are also well-known for their ability to produce medicinal compounds (De Silva et al. 2013, Degenkolb & Vilcinskas 2016). Thus, there have been several studies that have explored the production of novel compounds from endophytes of medicinal plants (Garcia et al. 2012, Nalini et al. 2014, Nath et al. 2015, Liu et al. 2016, Khan et al. 2017). A single study on the endophytic fungi of *R. stricta* has been carried out in Oman (Khan et al. 2015) and they identified two new enzyme inhibitory compounds: sorokiniol and radicinol and two known cyclic peptides (Khan et al. 2015, Ali et al. 2016). The aims of this study were therefore to identify endophytic fungi of *Rhazya stricta*, as well as to determine active secondary metabolites in the extracts of the isolated endophytic fungi. We focused on the antibacterial and antifungal properties of these extracts.

Materials & Methods

Collection of plant materials and isolation of endophytic fungi

Fresh healthy-looking leaves of *Rhazya stricta* were collected from Rawdat Khoraim, 25° 13′ 49″ N, 47° 10′ 15″ E, located 90 Km north east of Riyadh, Saudi Arabia. Samples were collected on 7 May 2015 and kept in clean plastic bags, in ice bags, returned to the laboratory and processed within 24 hours (Verma et al. 2007). In the laboratory, plant samples were washed with running tap water. The leaves were cut into segments (about 0.5 cm long). Segments were surface-sterilized by submersion in 95% ethanol for 1 minute, 2.5% sodium hypochlorite for 3 minutes, and 95% ethanol for 1 minute and completed by rinsing in sterile water three times (1 minute each). In each Petri-dish (9 cm in diameter), a total of four surface sterilized segments were evenly placed onto the surface of PDA plates supplemented with chloramephenicol for suppressing bacterial growth. The dishes were incubated at 28 C and hyphal tips growing out from the surface sterilized plant pieces were transferred to new PDA plates and preserved in cryotubes in 10% glycerol at -80 C in a deep freezer (Verma et al. 2007, Kharwar et al. 2008).

Morphological identification of endophytic fungi

Pure isolates were classified into morphotypes (*sensu* Lacap et al. 2003) according to the shape and color of the colonies. Fruiting morphotypes were identified based on their fruiting structures.

Molecular identification of Nothophoma multilocularis

Pure culture of the fungus was grown in YMG broth (4 g yeast extract, 10 g glucose, 10 g malt extract in 1 liter sea water) until sufficient mycelia was formed for DNA extraction. Total genomic DNA was extracted using Microbial DNA extraction kit (MOBIO; Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. LROR and LR7 primers were used for the amplification of partial LSU ribosomal DNA (Vilgalys & Hester 1990). Sequencing was made by Macrogen Inc., Korea. Details of the methods used are described in Abdel-Wahab et al. (2016). Sequences were assembled using Sequencher 4.2.2 (Gene Codes Corporation). Closest sequences were obtained using blast searches at GenBank. Sequences were aligned using ClustalX (Thompson et al. 1997) and manual adjustments of the sequences were carried out when necessary. Representatives of Pleosporaceae were used as out group (Fig. 1). Phylogenetic analyses were carried out using PAUP* v. 4.0b10 (Swofford 2002). Maximum-likelihood analysis (Felsenstein

1981) was performed using heuristic searches with the random stepwise addition of 100 replicates and tree bisection-reconnection (TBR) rearrangements. The optimal model of nucleotide substitution for the ML analyses was determined using hierarchical likelihood ratio tests as implemented in Modeltest 3.7 (Posada & Crandall 1998). The model selected as the best fit for LSU rDNA dataset was TrNef+I+G. Maximum-parsimony (MP) trees were obtained by 100 random addition heuristic search replicates using phylogenetic packages, and 1000 bootstrap replicates were performed employing 5 random addition heuristic searches. Bayesian analyses were performed by using PAUP v. 4.0b10 (Swofford 2002) and MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The model of evolution (SYM+I+G) was estimated by using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) were performed by Markov Chain Monte Carlo sampling (BMCMC). Five million generations were run in four chains with sampling every 100 generations, yielding 50000 trees, of which the first 12500 trees, representing the burn-in phase of the analyses, were discarded and the remaining trees used for calculating posterior probabilities (PP) in the majority rule consensus tree. Produced phylogenetic analyses were visualized using Njplot (Perrière & Gouy 1996). The alignment was deposited in TreeBASE (http://www.treebase.org) under the submission S21238.

Cultivation and extraction and natural product isolation

Fungal isolates were grown on PDA plates for two weeks. Mycelia with agar were cut into small cubes (*ca* 1 mm). Cubes were transferred aseptically to one Liter flask that contains 600 ml of YMG broth (10 gm yeast extract, 4 gm malt extract and 10 gm glucose in 1 liter of distilled water) and adjust pH at 5.5. The seeded flask was incubated at 28 C under stationary conditions until the concentrations of the glucose level reach 0.05%. The cultures were harvested and filtered through Whatman filter paper by vacuum filtration using pump and Büchner funnel. Filtrates were extracted using ethyl acetate in one to one ratio and then dried by rotary evaporator to give the crude extracts. Weights of the crude extracts were determined and were dissolved in dimethyl sulfoxide (DMSO) to give concentration 20 mg/ ml.

Test organisms

The pathogenic bacteria *Escherichia coli* (gram negative), *Staphylococcus aureus* (gram positive) and the yeast species, *Candida albicans* and the filamentous fungus *Aspergillus fumigatus* were used to carry out the bio-assay.

Antimicrobial activity

For antimicrobial evaluation, disk diffusion bioassay was performed. Plates of bacterial and fungal spore suspension were prepared using pour plate method. Five µl was added of the prepared DMSO solutions to each disk so that each disk has 100 µg of the crude extract. Plates were incubated at 37°C for bacteria and at 28°C for fungi and clear zone were measured in mm.

Isolation of metabolites

The crude extract of *Nothophoma multilocularis* gave the best results and was selected for further study and was grown on 10 L of the same medium structure under the same growth conditions. The resulted crude extracts were fractionated using silica gel columns. The weight of the crude extract was determined and 30 to 50-fold of that weight of silica gel was used. Crude extract was dissolved in one ml of acetone and mixed with one gram of silica and mixed and stirred until crude extract is totally mixed with silica gel and acetone is evaporated. Ethyl acetate is used as the solvent. Fractions were collected every 5 minutes. A total of 15 fractions were collected. Similar fractions were determined using TLC sheets and mixed. Bioassays of the fractions were carried out as described above. Active fractions were further fractioned using TLC sheets. Compounds in the active sub-fractions were determined using mass spectrometry (GC-MS).

Results & Discussion

Twenty-eight fungal isolates representing five species were isolated from 21 leaves (10 young and 11 old) of Rhazya stricta. These fungi include two species of Alternaria, Aspergillus sp.,

Nothophoma sp. and one sterile mycelia (Table 1). Khan et al. (2015) cultured five endophytic fungal isolates from 500 stem, root and leaf segments of *Rhazya stricta* from Oman. These five isolates represent three fungi namely: *Alternaria* sp., *Bipolaris sorokiniana* Shoemaker and *Phoma* sp. Their results are in harmony with our results in terms of low fungal diversity and *Alternaria* species were common to both studies. Compared with other studies in terrestrial ecosystems, the numbers of endophytes is very low (five taxa in this study, 35 from *Livistona chinensis* in Guo et al. (2000), 28 OTUs from *Bletilla ochracea* using molecular techniques in Tao et al. (2008).

The isolated fungi were tested against pathogenic microbes. Three species gave positive results, of which *Alternaria* sp. 1 (E-2-1) gave positive results against Gram positive (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli*). *Aspergillus* sp. (E-2-2) showed antimicrobial activity against bacteria and *Candida albicans*. *Nothophoma multilocularis* (E-2-5) gave best results against bacteria and fungi and was chosen for further study (Table 2).

Table 1 Endophytic fungi isolated from 21 leave segments of *Rhazya stricta*

Taxon	N	%
Alternaria sp.1 (E-2-1)	7	33.3
Alternaria sp. 2 (E-2-3)	3	14.3
Aspergillus sp. (E-2-2)	5	23.8
Nothophoma multilocularis (E-2-5)	7	33.3
Sterile mycelium (E-2-4)	6	28.6

N number of isolates.
% Frequency of occurrence.

Table 2: Bioassay results of the isolated fungi (the numbers are the clear zones in centimeter)

Taxon	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
Alternaria sp.1 (E-2-1)	0.8	1	negative	negative
Alternaria sp. 2 (E-2-2)	negative	negative	negative	negative
Aspergillus sp. (E-2-3)	0.9	1	1	negative
Nothophoma multilocularis (E-2-5)	1.7	1.5	negative	1
Sterile mycelium (E-2-4)	negative	negative	negative	negative

***Nothophoma* dataset:**

The LSU rDNA dataset consisted of 13 taxa of which seven are the *Nothophoma* and three belong to Pleosporaceae and used as the outgroup. The dataset includes 826 characters with 44 parsimony-informative characters. One most parsimonious tree was obtained after the search, all with a tree length of 79 steps, a consistency index of 0.7722, a retention index of 0.856 and a rescaled consistency index of 0.661. Maximum likelihood analysis produced one tree (–ln likelihood = 1570.98604). Bayesian analyses produced two phylogenetic trees of which one is shown in Fig. 1. MP and ML produced trees with similar topology to the one shown in Fig. 1. The new *Nothophoma* species nested within the clade containing the six other known species of *Nothophoma* with high statistical support 94/98/100 for MP/ML/Bayesian pp respectively. *Nothophoma multilocularis* clustered with *N. gossypiicola* and *N. macrospora* with weak to moderate bootstrap support 75/53 for P/ML respectively. Morphological characteristics and phylogenetic analyses of LSU rDNA sequences show that *Nothophoma multilocularis* (E-2-5) represent unknown fungus and it is described here as a new species. Similar phylogenetic tree topology and bootstrap support based on ITS and β-tubulin (TUB 2) of *Nothophoma* species was recently published (Bai et al. 2016).

Taxonomy

Nothophoma multilocularis Abdel-Wahab, sp. nov. Fig. 2

MycoBank number: MB821831; Facesoffungi number: FoF03436

Etymology – named for its multiloculate pycnidia.

Holotype – AUMC-12003-H.

Hyphae 2.5-6 µm thick, hyaline to yellow-brown, septate, immersed in the media. Pycnidia 175-1500 µm diameter, globose, subglobose or irregular in shape, stromatic, unilocule to multilocular or confluent with one to several long necks (up to 6), ostiolate, coriaceous to carbonaceous, black, superficial on or immersed into the agar. Necks 250-400 µm long, 80-130 µm wide, black, cylindrical with wide base. Pycnidial wall 38-80 µm thick forming *textura angularis*, one-layered, 8-18 cell layers, consists of polygonal cells with wide lumina 6-10 µm, dark-brown to black to outside and hyaline to inside; pycnidial centrum filled with pseudoparenchymatous cells that disintegrate with the development of the conidia. Conidiogenous cells 11-17 × 9-18 µm, phialidic, flask-shaped or polygonal, hyaline to yellow-brown. Conidia 9-20 × 3-4(5) µm ($\bar{x}$ = 14.9 × 3.9 µm, n = 50), unicellular, hyaline, smooth, cylindrical or clavate with rounded ends, with a few minutes polar guttules, conidium length/width ratio are 4–5.5/1 ($\bar{x}$ = 3.84, n = 50). Chlamydospores 10-16 µm in diam., globose, subglobose to polygonal, brown to dark-brown, in chains or in large masses of cells 65-130 × 22-45 µm. NaOH spot test: negative.

Culture characteristics – Colonies on Oatmeal Agar (OA), covering 40-45 mm diameter after 7 days in the dark at 25 C; circular with complete edge, olive brown, the first 0.5 cm to the margin is hyaline in color, heavily sporulating with fertile stromatic pycnidia, flat, slimy growth; reverse olive brown. Colonies on PDA, covering 28-30 mm diam. In Petri-dishes after 7 days in the dark at 25 C; circular with complete edge, olivaceous-brown, heavily sporulating with fertile stromatic pycnidia, flat, slimy growth; reverse olive brown. Colonies on CMA, covering 32-35 mm diam. after 7 days in the dark at 25 C; circular with complete edge, light-brown, poor sporulation, flat; reverse light-brown.

Material examined – SAUDI ARABIA, Riyadh City, Rawadat Khoraim (25° 13′ 49″ N, 47° 10′ 15″ E), an endophyte of the healthy-looking leaves of the medicinal plant *Rhazya stricta*, 7 May 2015, Mohamed S. Hodhod (AUMC-12003-H, holotype), ex-type living cultures AUMC-12003.

Notes – *Nothophoma* Q. Chen & L. Cai, typified by *N. infossa* (Ellis & Everh.) Q. Chen & L. Cai. was recently established to accommodate five *Phoma* species clustered in a monophyletic clade in Didymellaceae (Chen et al. 2015), Sordariomycetes (Maharachchimbura et al. 2016). Another *Nothophoma* species, *N. macrospora* Valenzuela-Lopez, Stchigel, Cano & Deanna A. Sutton, was described from a human clinical specimen (Crous et al. 2016). The first report of *Nothophoma quercina* (Syd. & P. Syd.) Q. Chen & L. Cai on *Ulmus* was provided by Tibpromma et al. (2017). Phylogenetic analyses based on LSU rDNA placed the new species along with the six species of Nothophoma *with* high statistical support 94/98/100 for MP/ML/Bayesian pp respectively. *Nothophoma multilocularis* clustered with *N. gossypiicola* and *N. macrospora* (Fig. 1). The three species produce conidia over 10 µm in length (Table 3). *Nothophoma multilocularis* differs from *N. gossypiicola* by its large pycnidia (175-1500 µm vs. 100-250 µm diam), the number of necks (up to 6 vs 0–1), larger conidiogenous cells (11-17 × 9-18 µm vs. 5-8 × 5-8 µm) and conidia (9-20 × 3-4(5) µm vs. 10-12.5 × 2.5-3.5 µm). *Nothophoma gossypiicola* is a well-known cause of leaf spots and stem cankers on cotton, *Gossypium* spp. (De Gruyter 2002). *Nothophoma multilocularis* differs from *N. macrospora* by its large pycnidia (175-1500 µm vs. 100-300 µm diam.), wider pycnidial wall (38-80 µm vs. 15-25 µm) and conidia (9-20 × 3-4(5) µm vs. 10-15 × 2.5-3.5 µm). Conidia in *N. multilocularis* are unicellular, while in *N. macrospora* they are 0-2 septate.

Natural products of Nothophoma multilocularis:

Fifty-five bioactive chemical compounds were identified from the crude extracts of Nothophoma multilocularis using GC-MS. Ten major bioactive compounds were recorded namely: Di-n-octyl phthalate representing 53.98 % of the crude extract, 2-Allyl-3,4-dimethoxybenzaldehye, (10.26 %), Maltol (9.45 %), Cetene (2.73 %), 1-Tetradecene (2.07 %), E-15-Heptadecenal (2.06 %), 2,5-Cyclohexadien-1-one (1.88 %), 1-Octadecene (1.36 %), Diethyldithiophosphinic acid (1.17 %) and Phenol, 2,4-di-t-butyl-6-nitrophenol (1.07 %).

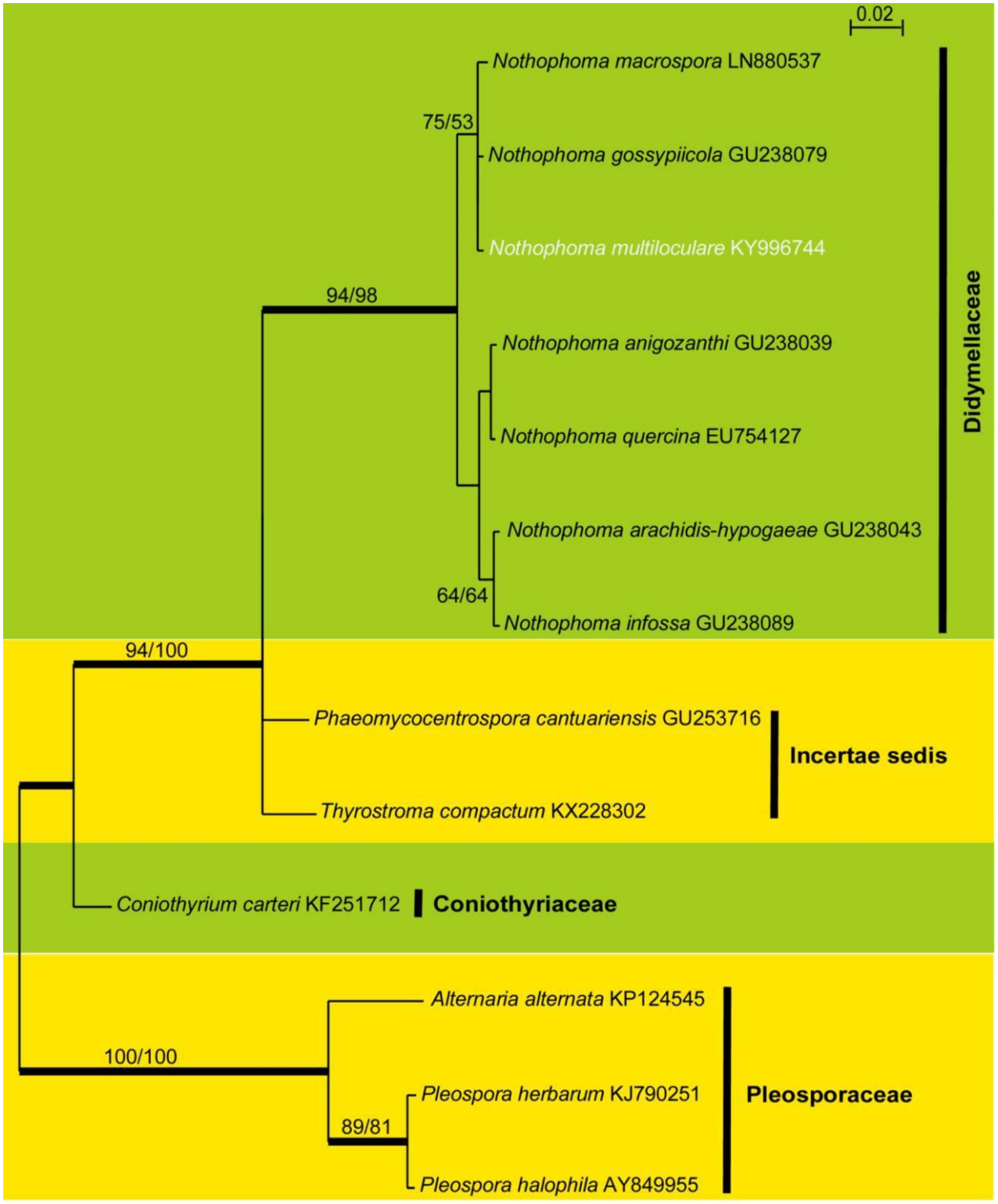


Fig. 1 – Phylogenetic relationships of the new species with other *Nothophoma* species. Phylogenetic tree derived from Bayesian analysis, based on the nucleotide sequences of LSU rDNA. The numbers on the nodes indicate pp values ≥ 95% (in bold), MP bootstrap and MP bootstrap values ≥ 50%. The tree is rooted to representatives of Pleosporaceae. New species is in white.

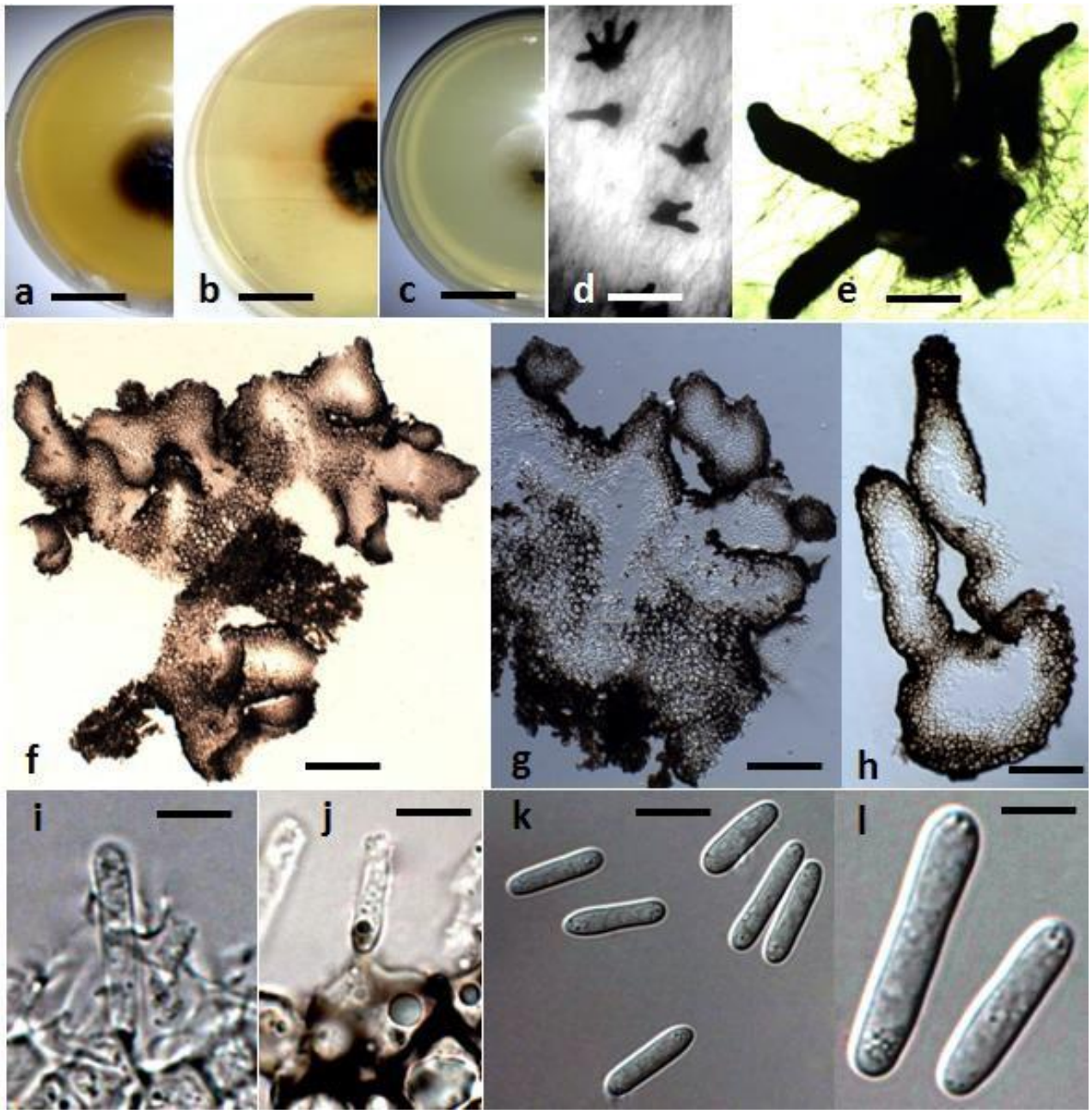


Fig. 2 – ***Nothophoma multilocularis*** from the culture **(ex-type)**. **a-c** Cultures from above incubated for 6 days on: **a** OA, **b** PDA, **c** CMA. **d, e** Pycnidia. **f-h** Vertical sections through stromatic pycnidia. **i, j** Conidiogenous cells and young conidia. **k, l** Conidia. Scale bars: a-c = 2 cm, d = 1000 µm, e, f = 200 µm, g, h = 100 µm, i-k = 10 µm, l = 5 µm.

These compounds showed strong antimicrobial activity in combination. Di-n-octyl phthalate was the major compound in the active fraction representing 53.98 % (Table 4). Phthalates are used in the manufacture of a variety of plastics and coating products. Phthalates were recorded from various biological resources including plants (Duc et al. 2007). Fatty foods such as milk, butter, and meats are found to be the main sources of natural bis(2-ethylhexyl) phthalate and other phthalates (Kohn et al. 2000). 1, 2-Benzenedicarboxylic acid bis (2-ethylhexyl) phthalate has been isolated from a marine alga, *Sargassum weightii*, from the seeds of *Ricinus.*

Table 3 Morphological comparison of *Nothophoma* species:

Species	Conidiomata	Conidiogenous cells	Conidia	Chlamydospores	Culture characteristics	Host
N. anigozanthi[1]	Pycnidial, solitary or aggregated, olivaceous buff 70-130 µm diam, turn black with age, 155-280 × 140-230 µm. Ostioles 1-4(-6) on long neck. Wall 3-6-layered 16-41 µm thick.,	Phialidic, hyaline, ampulliform to doliiform, 5-9 × 4.5-7.5 µm.	Ellipsoidal, aseptate, 3.5-5 × 1.5-2.5 µm, with several minute guttules.	Absent	On OA 40-50 mm, after 7 d, olivaceous, reverse concolourous. NaOH spot test: a luteous discoloration on MEA, change to dull green.	Parasitic on *Anigozanthus* spp.
N. arachidis-hypogaeae[2]	Pycnidial 80-200 µm in diam, globose to bottle-shaped, solitary or in raws, not confluent, papillate, citrine-honey then olivaceous to black. Wall made up of 3-5 layers, outer layers pigmented.	Globose to bottle-shaped, 3-8 × 3-7 µm.	Oblong to ellipsoidal without or with two minute polar guttules, aseptate, 3.2-5.2 × 1.8-2.4 µm.	Absent	On OA 47-48 mm, grey olivaceous reverse olivaceous. NaOH spot test: on MA a slight reddish discoloring occurs.	Parasitic on *Arachis hypogaea*
N. gossypiicola[3]	Pycnidial 100-250 µm in diam, globose to subglobose, solitary or confluent, without or with one non-papillate ostiole, honey, later olivaceous to black Walls made up of 3-10 layers of cells.	Globose to bottle-shaped, 5-8 × 5-8 µm.	Ellipsoidal with several minute guttules, aseptate, 10-12.5 × 2.5-3.5 µm.	globose to elongate, usually in chains, olivaceous with greenish guttules, 8-12 µm diam.	On OA 47-55 mm, after 7 d, dull green to olivaceous, reverse olivaceous. NaOH spot test: negative.	Parasitic on *Gossypium* spp.
N. infossa[4]	Pycnidial mostly solitary, subglobose to elongated, 190-250 × 140-180 µm. Ostioles mostly single 40-75 µm diam. Wall 5-9 layers, 28.5-55 µm thick.	Phialidic, hyaline, simple, smooth, flask-shaped, 5.5-8 × 5-5.5 µm.	Ovoid, thin-walled, hyaline but incidently brown, aseptate, 4.5-6 × 2.5-3.5 µm, without or with minute polar guttules.	Honey to cinnamon, dictyosporous or phragmosporous, solitary or forming long chains, 18-32 × 11.5-17 µm	On OA 45-55 mm, olivaceous, reverse gray.	Parasitic on *Fraxinus pennsylvanica*
N. macrospora[5]	Pycnidial pyriform, dark-brown, 2-3 necks, 100-300 µm in diam. Wall 3-5 layers, 15-25 µm	Enteroblastic, phialidic, globose to flask-shaped, hyaline, 5-10 µm diam.	Cylindrical or clavate, 0(-2)-septate, 10-15 × 2.5-3 µm, guttulate.	Absent	On OA 30 mm, after 7 d, olive brown, reverse concolorous.	Isolated from human clinical specimen.
N. multilocularis[6]	Pycnidial globoe, stromatic, uniloculate to multiloculate or confluent with up to 6 long necks diam. Wall 38-80 µm thick, 8-18 cell layers.	Phialidic, flask-shaped or polygonal, hyaline to yellow-brown 11-17 × 9-18 µm.	Unicellular, hyaline, with a few minute polar guttules, cylindrical or clavate 9-20 × 3-4 µm.	10-16 µm in diam, globose, subglobose to polygonal, brown to dark-brown, in chains, or in large masses 65-130 × 22-45 µm.	On OA 40-45 mm diam, after 7d, olive brown, reverse olive brown. NaOH spot test: negative.	An endophyte of *Rhazya stricta*.
N. quercina[7]	Pycnidial solitary, globose, 65-130 × 95-200 µm, with single non-papillate ostiole. Wall 8.5-14.5 µm.	Phialidic, hyaline, simple, smooth, doliiform to ampulliform, 3.5-5× 3-4 µm.	Subglobose to oval or obtuse, aseptate, 5.5-7.5 × 3-4 µm, with 0-2 minute guttules, hyaline but brown at maturity.	NA	On OA 55-68 mm diam, after 7d, greenish olivaceous, reverse concolourous.	Parasitic on *Quercus* sp.

[1]Chen et al. 2015, [2] De Gruyter et al. 1993, [3] De Gruyter 2002, [4] Aveskamp et al. 2009, [5] Crous et al. 2016, [6] This study, [7]Aveskamp et al. 2010.

Key to species of *Nothophoma*

1. Conidia below 10 µm in length.....2
1. Conidia over 10 µm in length.....3
2. Parasitic on *Anigozanthus* spp.; pycnidia olivaceous buff, 70-130 µm diam, turn black with age, 155-280 × 140-230 µm, with 1-4(-6) long necks; conidia 3.5-5 × 1.5-2.5 µm, aseptate, ellipsoidal..... *N. anigozanthi*
2. Parasitic on *Arachis hypogaea*; pycnidia citrine-honey then olivaceous to black, 80-200 µm in diam, solitary or in rows, not confluent; conidia 3.2-5.2 × 1.8-2.4 µm, aseptate, oblong to ellipsoidal .....*N. arachidis-hypogaeae*
2. Parasitic on *Fraxinus pennsylvanica*; pycnidia mostly solitary, 190-250 × 140-180 µm, with single ostiole; conidia 4.5-6 × 2.5-3.5 µm, aseptate, ovoid, hyaline but incidentally brown .....*N. infossa*
3. Parasitic on *Gossypium* spp.; pycnidia 100-250 µm in diam, honey turn to olivaceous black with age, without or with non-papillate ostiole; conidia 10-12.5 × 2.5-3.5 µm, aseptate, ellipsoidal..... *N. gossypiicola*
3. Isolated from human clinical specimen, pycnidia 100-300 µm in diam., dark-brown, with 2-3 necks; conidia 0(-2)-septate, 10-15 × 2.5-3 µm, cylindrical or clavate..... *N. macrospora*
3. Endophyte of *Rhazya stricta*; pycnidia 175-1500 µm diam, black, with 1-6 necks; conidia 9-20 × 3-4 µm, aseptate, cylindrical or clavate ..... *N. multilocularis*

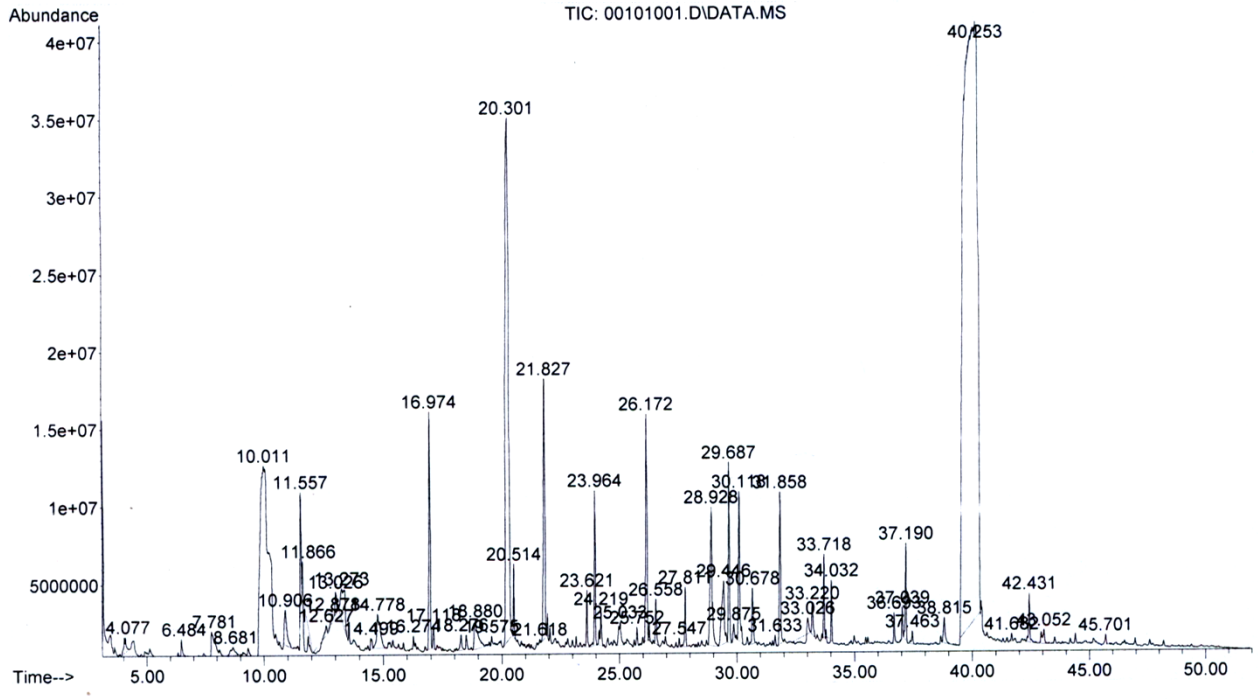


Fig. 2 – GC-MS chromatogram of the active fraction of the ethyl acetate of *Nothophoma multilocularis*.

communis and leaves of other plants and showed antibacterial effect (Sasty & Rao 1995, Wei & Wang 2006, Ruan et al. 2006, Sani & Pateh 2009). Phthalates were reported to have antimicrobial and other pharamacological activities. Bis (ethyl hexyl) phthalate reported from *Streptomyces bangladeshiensis* showed antimicrobial activity against gram positive bacteria and some pathogenic fungi (Al-Bari et al. 2006). Phathalates showed anti-inflammatory (24) and anticancer activity (Nguyen et al. 2007,

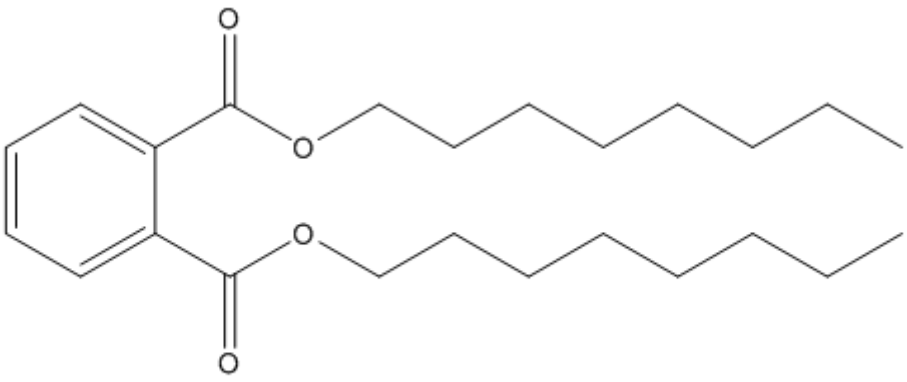
Mavar et al. 2008). The essential oil of *Leea indica* (Burm. F) Merr flowers showed phthalic acid esters (95.6%) as major constituents and had good antibacterial and antifungal activity (Srinivasan et al. 2009).

Table 4 Major natural products compounds identified in the ethyl acetate extract from the culture filtrate of *N. multilocularis* by GC-MS:

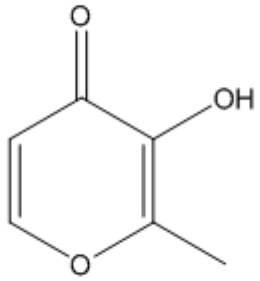
Peak No.	R-Time	Name of the compound	Molecular formula	Molecular weight	Area %	Activity
5	10.01	Maltol	$C_6H_6O_3$	126.11	9.45	Antifungal
16	16.97	1-Tetradecene	$C_{14}H_{28}$	196.37	2.07	Antimicrobial
21	20.3	2-Allyl-3,4-dimethoxybenzaldehyde	$C_{11}H_{12}O_3$	192.21	10.26	New compound
24	21.82	Cetene	$C_{16}H_{32}$	224.43	2.73	Antioxidants
26	23.96	Phenol, 2,4-di-t-butyl-6-nitrophenol	$C_{14}H_{21}NO_3$	251.32	1.07	Antimicrobial, anticancer
30	26.17	E-15-Heptadecenal	$C_{17}H_{32}O$	252.44	2.06	Antimicrobial
34	28.92	2,5-Cyclohexadien-1-one,2,6-bis (1,1-dimethylethyl)-4-methylene-	$C_{15}H_{22}O$	218.33	1.88	
35	29.44	Cyclo (L-Leucyl-L-Prolyl)	$C_{11}H_{18}N_2O_2$	210.27	1.17	Antifungal and anti-aflatoxins production
38	30.12	1-Octadecene	$C_{18}H_{36}$	252.48	1.36	
51	40.25	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390.56	53.98	Antimicrobial, antioxidant Plasticizer, cosmetics

2-Allyl-3, 4-dimethoxybenzaldehyde (10.26 %) is the second major compound in the active fraction of the crude. This compound seems to be a new compound and we will do more analytical work on it. Maltol (9.45 %) is the third major compound in the crude extract. Maltol is a naturally occurring organic compound that is used primarily as a flavor enhancer and has excellent anti-oxidative activity (Hong et al. 1992). Maltol was isolated from the bark of larch tree, pine needles and found also in roasted malt. However, maltol has rarely been described as a microbial metabolite (Cunningham & Pickard 1985).

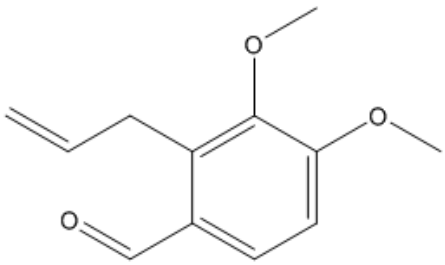
Chemical structure of the 10 major compounds



Di-n-octyl phthalate



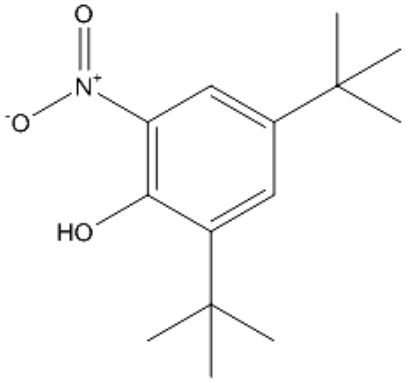
Maltol



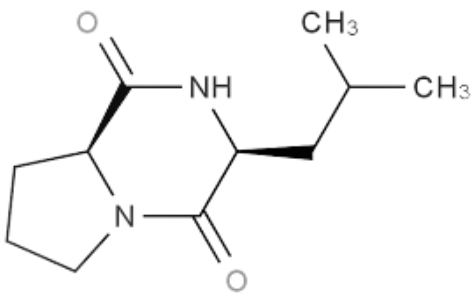
2-Allyl-3,4-dimethoxybenzaldehyde

1-Tetradecene

Cetene

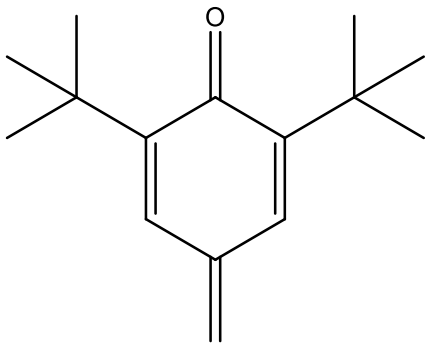


2,4-di-t-butyl-6-nitrophenol



Cyclo (L-Leucyl-L-Propyl)

E-15-Heptadecenal



2,5-cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-methylene-

1-Octadecene

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