Metabolite Profiling of the Extracts of Endophytic Fungi of Entomopathogenic Significance, *Aspergillus flavus* and *Nigrospora sphaerica* Isolated from Tropical Tree Species of India, *Tectona grandis* L.

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Abstract

India needs newer group of biopesticides. Biopesticides are part of the insecticide field, their market is increasing. Biopesticides are secondary metabolites include thousands of alkaloids, terpenoids, phenolics and minor secondary chemicals derived from plants, microbes, animals, and certain minerals. Several endophytic microbes are known to have anti-insect properties. Endophytes are microbes which colonize living, internal tissues of plants without causing any harm to their host. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. Endophytic fungi, A. flavus and N. sphaerica isolated from teak leaves produced phytochemicals such as Duroquinone, Adamantine derivative, Dodecanoic acid, tetradecanoic acid, pentadecanoic acid and Myristic acid which are reported to have insecticidal activity. Hence, the extracts of A. flavus and N. sphaerica may be considered to use as novel insecticides.

Keywords: Endophytic fungi, *Aspergillus flavus*, *Nigrospora sphaerica*, secondary metabolites, endomopathogenic significance, *Tectona grandis*

Introduction

Ecological damage provoked by extensive use of synthetic insecticides augment natural product research for the discovery of powerful, selective, and safe alternatives (Strobel and Daisy, 2003). Many synthetic agricultural agents have been targeted for removal from the market, because of profound harmful effects on human health and environment. Thus, perhaps endophytic fungi could serve as reservoir of untapped biologically based compounds that may present alternative ways to control farm pests and pathogens (Demain, 2000). Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate negative effects. They reside inside the tissues of nearly all healthy plants. They are synergistic to their host and atleast some of them are thought to be useful to the plant by producing special substances, such as secondary metabolites, that prevent the host from being attacked successfully by fungi and pests (Thongchai *et al*., 2003). One interesting finding consisted in the discovery of peramine, which was toxic to insects without any harmful impact on mammals. This secondary metabolite was characterized in cultures of *Neotyphodium coenophialum*, *N. lolli*, *Epichloë festucae* and *E. typhina* associated with tall fescue, ryegrass and other grasses (Dew *et al.*, 1990). Nodulisporic acids were isolated from a *Nodulisporium* sp. endophyte in *Bontia daphnoides*. They were found to exhibit potent insecticidal properties against the larvae of the blowfly (Demain, 2000). Another endophytic fungus, *Muscodor vitigenus* isolated from *Paullina paullinioides*, was found to yield naphthalene as its major product. Heptelidic acid and hydroheptelidic acid, from *Phyllosticta* sp. an endophytic fungus of *Abies balsamea*, have been shown to be toxic to spruce bud worm (*Choristoneura fumiferana*) larvae (Calhoun *et al.*, 1992). Being poorly investigated, endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential.

Hence, the objective of this study is to identify bioactive compounds in the extracts of endophytic fungi with entomopathogenic significance isolated from *T. grandis* (teak) leaves using GC-MS technique. This work will help to identify the compounds of insecticidal potential. The endophytic fungi, *Aspergillus flavus* (Indian Type Culture Collection - ITCC 9080.13) and *Nigrospora sphaerica* (ITCC 9079.13) were subjected as biological sources of the study. Our preliminary studies on the efficacy of these endophytic fungi against teak defoliator, *Hyblaea purea* gave promising results. This is an eye opening for us to identify the secondary metabolites in the extracts of these endophytic fungi for insecticidal properties.

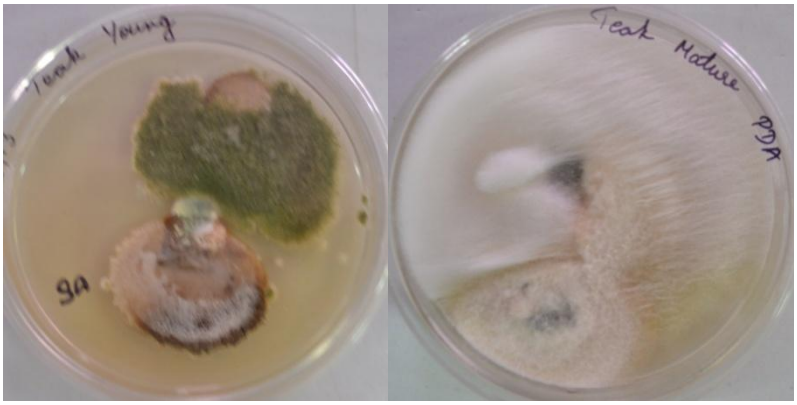
Materials and Methods

Collection of Plant Samples

The leaves of *T.grandis* were collected from Mudumalai Wildlife sanctuary (11° 34′ 37.60″ N latitude and 76°34′ 05.19″E longitude; 907m MSL), Nilgiris District; Ramapuram village (12° 27′ 58.0″ N latitude and 79°45′ 41.71″E longitude; 65m MSL) in Melmaruvathur Taluk and Mettukadu village (12° 54′ 56.2″ N latitude and 79°43′ 38.1″E longitude; 85M MSL) in Walajabad Taluk in Kanchipuram District of Tamilnadu, India. At each location, trees free from insect and disease infestation were selected and marked. Healthy leaves from these healthy trees were collected and processed separately within 48 h of collection.

Isolation and Identification of Endophytic Fungi

The leaves were washed thoroughly in running water and segments of 1 cm^2 were cut from the midrib portion of each leaf and surface sterilized by immersing in 70% ethanol for 1 min, followed by 4% sodium hypochlorite (v/v) for 2 min, and finally washed in sterile water for 1min (Suryanarayanan *et al*., 1998). Each segment from each individuals were placed in Petri dishes containing potato dextrose agar (with chloramphenicol 150 mg l–1). Two leaf segments were plated in each Petri dish, the dishes were sealed with parafilm and incubated in an incubator at 26 ± 1°C for 21 days. The efficacy of the sterilization procedure was ascertained with the method of Schulz *et al*. (1998). In addition, 10 ml of the last rinsing water were centrifuged for 10 min at 5000 rpm. The supernatant was removed and added 500 µl sterilized water in the centrifugal tube; 100 µl of this volume were then plated onto PDAS. The surface sterilization was validated because no mycelial growth occurred. The fungi that grew out from the segments were isolated and identified. Pure fungal cultures of the endophytic isolates were obtained by the hyphal tip method in test tube slands. All fungal isolates were numbered and sent to ITCC, IARI, New Delhi for confirmation. Simultaneously, the isolates were stored in 15% (v/v) glycerol at −80°C in deep freezer (VWR Scientific) as spores and mycelium for further study.



Aspergillus Flavus (ITCC 9080.13) **Nigrospora Sphaerica (ITCC 9079.13)**

Mass Culture and Preparation of Fungal Extracts

The endophytic fungi were grown in 2 litre Erlenmeyer flasks containing 500 ml of PDB medium supplemented with soytone (Pinkerton and Strobel, 1976) and incubated for 21 days. After 3 weeks of still culture at 26 °C, the culture fluid was passed through four layers of cheese cloth to remove solids and extracted with organic solvent. The extraction and isolation procedure followed was that of Strobel *et al.* (1996). After methylene chloride extraction, the organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 35 °C using rotary vacuum evaporator (Heidolph VV2000) followed by vacuum concentrator (Savant, SC 110).

The concentrated extract was subjected to freeze drying in a lyophilizer (Virtis) till dry powder was obtained. Finally the extracted powder was suspended with methanol at the concentration of 100mg/ml (w/v) followed by filtration through Varian Bond Elute C18 solid phase extraction to remove impurities. This is treated as stock. Different concentrations viz., 250, 500, 750 and 1000 ppm were prepared from the stock solution for bioassay studies. 1µl of the stock solution was employed for GC-MS-MS analysis.

GC-MS –MS Conditions

The GC-MS-MS analysis of the extracts was carried out using Varian 4000 Ion trap GC/MS/MS with Fused silica 15m x 0.2 mm ID x 1µm of capillary column. The instrument was set to an initial temperature of 110 ºC, and maintained at this temperature for 2 min. At the end of this period the oven temperature was rose up to 280 ºC, at the rate of an increase of 5 ºC/min, and maintained for 9 min. Injection port temperature was ensured as 250 ºC and Helium flow rate as 1 ml/min. The ionization voltage was 70eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45-450 (m/z). Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS-MS compounds present in the extracts were identified.

Identification of Bioactive Compounds

Interpretation on mass-spectrum GC-MS-MS was conducted using the database of National Institute Standard and Technology (NIST) having more 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library. The name, molecular weight and molecular formula of the extracts samples were ascertained.

Bioassay: Bioefficacy of extracts made from culture filtrates of *A. flavus* and *N. spharica* was evaluated against teak defoliator, *Hyblea purea* and Ailanthus defoliators, *Atteva fabriciella* and *Eligma narcissus*. Leaf discs (4cm dia) of *T. grandis* and *Ailanthus excelsa* were used for bioassay tests, after washing them with tap water. The leaf discs were sprayed with 250, 500, 750 and 1000ppm concentrations of each extracts for twenty seconds; air dried at room temperature and kept in petri plates (9cm dia). The pre starved (24 h) larvae of *H. purea*, *A. fabriciella* and *E. narcissus* were allowed to feed on the treated leaf discs of *T. grandis* and *A. excelsa* respectively till 96 hours. Each treatment was replicated five times and twenty insects in each replicate with one control were maintained. Larval mortality and pupal deformities were recorded during the course of the experiment. The data collected was subjected to analysis of variance using statistical software SPSS version 16.0.

$$\text{Per cent larval mortality} = \frac{\text{Number of dead larvae}}{\text{Total number of treated larvae}} \times 100$$

Results and Discussion

The endophytic fungi isolated were confirmed as *Aspergillus flavus* (Indian Type Culture Collection - ITCC 9080.13) and *Nigrospora sphaerica* (ITCC 9079.13) (Dr. T. Prameela Devi, Principal Scientist (ITCC), IARI, New Delhi, India). The fungi were mass cultured and extracted as the method described above.

Teak, *T.grandis* is one of the most important tropical hardwood forest species in the international market because of its high quality timber. Hence, it has been deployed in large scale plantations in Kerala and Tamilnadu. The most serious insect pest of teak is teak defoliator, *H.puera*. The biomass loss due to *H. purea* is 0.05 ton/tree. Similarly, *A. excelsa* is also an economically important indigenous tree species in India for safety matches industry. *A. excelsa* is generally considered to be the best Indian tree species for match splints. *A. excelsa* is generally grown in the bunds of the farmlands in Tamilnadu and Kerala states. Increased demand of *A. excelsa*, steady depletion of match wood resources, can be tackled only by raising new matchwood plantations. The most destructive defoliators of *A. excelsa* are *A. fabriciella* and *E. narcissus.* The biomass loss due to Ailanthus defoliators is 4 tons/ha. Synthetic organic insecticides have emerged as chief major tools of pest management. However, due indiscriminate use of synthetic chemicals, insect pests have developed resistance to insecticides, resurgence of secondary pests, reduction in the population of natural enemies, and harmful residues in food, feed and fodder. These concerns have led to the surge of alternative pest control technologies. The pesticide formulations based on chemicals from living organisms have attracted particular attention because of their specificity to insect pests, their biodegradable nature and a potential for commercialization. Several endophytic microbes are known to have anti-insect properties.

They colonize living tissues of plants without causing any harm to their host. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites. Hence, an attempt has been made to screen endophytic fungi isolated from tropical tree species, *T. grandis* for insecticidal compound to develop novel insecticide. The data summarized on table 1 represents the mortality of *H. puera, A. fabriciella* and *E. narcissus* larvae varied from 15 to 65 percent at different concentrations of extracts. The extracts of *A. flavus* and *N. sphaerica* at 1000 ppm showed high mortality with 65 and 62 percent respectively in *H. purea* followed by 59 and 56 percent in *E. narcissus* and 46 and 42 percent in *A. fabriciella*. The percent mortality was increased with increase in concentration and found statistically significant. This imposed us to screen these extracts to characterize secondary metabolites of insecticidal properties through GC/MS/MS analysis.

Table 1: Percent Larval Mortality of *H.puera, A. fabriciella* and *E. narcissus* Against Culture Filtrate Extract of *A. flavus* And *N.sphaerica*

Endophytic fungal extracts	Insect pests	Mortality %			
		250ppm	500ppm	750ppm	1000ppm
A. flavus	*H. purea*	28.33±11.02^{cd}	35.00±6.24^{e}	49.67±10.60^{d}	65.67±5.03^{d}
N.sphaerica		27.33±11.85^{cd}	31.67±15.31^{bc}	44.67±6.43^{cd}	62.33±4.62^{d}
A. flavus	*A. fabriciella*	18.33±5.86^{b}	29.00±9.54^{b}	32.33±2.52^{b}	46.84±0.89^{b}
N.sphaerica		15.00±4.36^{b}	25.33±17.21^{bc}	35.67±15.95^{b}	42.21±0.14^{b}
A. flavus	*E. narcissus*	24.67±6.35^{c}	28.67±11.72^{cde}	46.00±8.72^{d}	59.22±9.50^{c}
N.sphaerica		21.33±1.15^{c}	26.67±7.02^{bcd}	44.00±3.46^{cd}	56.33±3.06^{c}
Control	*H. purea*	4.67±0.58^{a}	4.67±0.58^{a}	4.67±0.58^{a}	4.67±0.58^{a}
	A. fabriciella	4.00±2.42^{a}	4.00±2.42^{a}	4.00±2.42^{a}	4.00±2.42^{a}
	E. narcissus	6.33±1.15^{a}	6.33±1.15^{a}	6.33±1.15^{a}	6.33±1.15^{a}

All values are mean ± SD of five replicates with 20 insects in each replicate (total 100 insects) values followed by the same alphabets are not significantly different at P<0.05 (DMRT).

The GC/MS/MS analysis of extracts of *A. flavus* and *N. sphaerica* culture filtrates (Fig 1 and 2) gave us thirty one and eleven major compounds respectively (Table 2 and 3). All compounds identified by GC/MS/MS screening were assessed for their insecticidal property using physico-chemical property calculations according to Tice Rules. As per Tice rule compounds are more likely to have properties of insecticide if molecular weight is within ≥ 150 and ≤ 500; theoretical logarithm of the noctanol/water partition coefficient (log P), is less than or equal to 5.0; hydrogen bond acceptor is within 1-8; hydrogen bond donar is less than or equal to 2 and the number of rotatable bond is less than or equal to 12. The compounds those are strictly following the Tice rules are considered as anti-insect compounds (Sanjayan and Praveena, 2013). Seventeen compounds from the extracts of endophytic fungi, *A. flavus* and seven compounds from *N. sphaerica* were observed strictly follow Tice rule. The presence of phytochemicals such as duroquinone, naphthelene, lauric acid, adamantine derivatives and amylmetacresol might be the reason for insecticidal activity of *A. flavus* and *N. sphaerica*. Compunds namely, Dodecanoic acid (Lauric acid) (18.02%), Tricyclo(4,3,1,1,(3,8) undecane 1- bromo (Adamantine derivative) (9.56%) in *A. flavus* extract and Benzoic acid -2(methylthio methyl ester (25.04%), 2,5,cyclohexadiena-1,4-dione, 2-(1,1-dimethyl) (Duroquinone) (17.81%), 2-(2-cyanoethyl) 3-isopropyl 4 and 5-cyanoisoxazolidine (9.19%) in *N. Sphaerica* extracts are recorded as major compounds which are not reported in endohytic fungal extracts earlier, might have shown promise to use as a source of insecticide. Hence, detailed study with respect to aforementioned compounds will be an insight into develop novel insecticide. Senthilkumar *et al*. (2011) reported tetradecanoic acid, dodecanoic acid and n-hexadecanoic acid in the extract of heads space of *Aspergillus versicolar. D*odecanoic acid and tetradecanoic acid was reported from *P. chrysogenum* (Griffith *et al*., 2007). Pentadecanoic acid and Myristic acid were reported from *Mortierella alpine* (Wang *et al.,* 2005). They also reported the antimicrobial activities of these compounds. Hexadecanoic acid and octadecanoic acid methyl ester were mainly found in endophytic fungi obtained from five Thai medicinal plants which are commonly used in Thai traditional medicines (Theantana *et al*., 2012). Dodecene was obtained as a major compound from an endophytic fungus, *Fusarium solani* isolated from *Taxus baccata* bark (Tayung *et al*., 2011). Hexadecene was identified as an extract of fungi *Monochaetia kansensis.* It was reported that various antimicrobial compounds were isolated from *Colletotrichum gloeosporioides (*Aoyagi *et al*., 2008*).* Naphthalene is used as an antimicrobial, insecticide, insect repellent, anthel-minthic and vermicide.

The production of insect-repellent naphthalene by endophyte *M. vitigenus* was recorded by direct gas chromatography/mass spec-trometry (Daisy *et al*., 2002). The production of naphthalene by endophytic fungi may be related to restrict the insect infestation on their hosts.

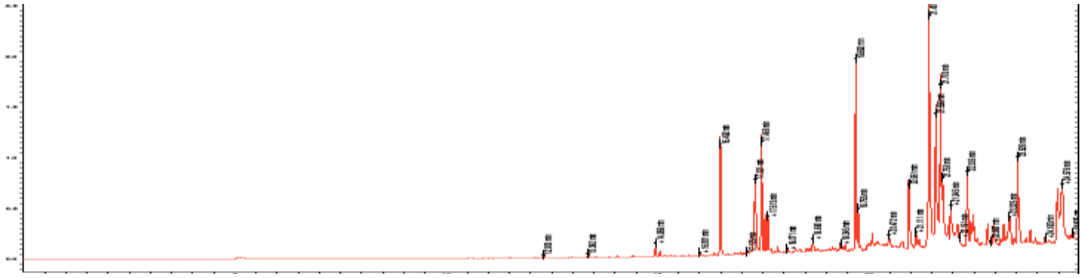


Fig. 1 GC/MS/MS Spectrum of Extracts of Endophytic Fungus, A. Flavus

Table 2 Chemical Composition of the Extract of *A. flavus* (GCMSMS analysis)

Constituent	Rt	%	MF	MW	Log P	HD	HA	RB
2,5,cyclohexadiena-1,4-dione, 2-(1,1-dimethyl) (Duroquinone)	12.301	6.54	$C_{10}H_{12}O_2$	164	2.2	0	2	0
1,4-dimethyladamantane (1, alpha, 3 beta, 4 beta)	12.303	Tr	$C_{12}H_2O$	164	4.5	0	0	0
Naphthalene, 1,2,3,4, tetra	13.361	4.43	$C_{11}H_{14}$	146	3.7	0	0	0
1,2,4,5,-tetrakis (trimethyl siloxy) benzene	13.534	6.77	$C_{18}H_{38}O_4Si_4$	430	2.1	0	4	2
Myristic acid, 2,3, bis(trimethylsiloxy) propyl ester	13.542	Tr	$C_{23}H_{50}O_4Si_2$	446	10.23	0	4	20
Thymolphthalein	13.561	Tr	$C_{28}H_{30}O_4$	430	6.8	2	4	4
2-(3-hydroxy propyl) piperidine.	13.864	2.29	$C_8H_{17}NO$	143	1.7	0	2	2
Leucinocanine	13.875	1.36	$C_{17}H_{28}N_2O_2$	292	2.1	0	4	2
Talsutin	13.932	2.14	$C_{16}H_{14}N_2O_6S$	362	1	3	6	5
1-Tetradecanol (Myristyl alcohol)	14.958	3.06	$C_{14}H_{30}O$	214	6.2	1	1	12
Eicosane	15.058	5.68	$C_{20}H_{42}$	282	10.4	0	0	17
Pentadecane	15.067	Tr	$C_{15}H_{32}$	212	7.7	0	0	12
Nonadecane	15.082	Tr	$C_{19}H_{40}$	268	9.9	0	0	16
Pumiliotoxin C	15.187	Tr	$C_{13}H_{25}N$	195	3.7	1	1	2
Pulegone	15.238	Tr	$C_{10}H_{16}O$	152	2.8	0	1	0
Calacorene	15.570	Tr	$C_{13}H_{16}$	172	4.1	0	0	0
Phytol	15.880	Tr	$C_{20}H_{40}O$	296	8.2	1	1	13
Solanocapsin	15.892	Tr	$C_{27}H_{46}\ N_2O_2$	430	5	3	4	0
Digitotoxigenin	15.923	Tr	$C_{23}H_{34}O_4$	374	2.6	2	4	1
N, methyl-m-hemipimide	16.007	6.24	$C_{11}H_{11}NO_4$	221	0.8	0	4	2
9H-carbazole,9-(1-methyethy)	16.274	6.51	$C_{15}H_{15}N$	209	3.6	0	1	0
Triteterracontane	16.335	2.3	$C_{43}H_{88}$	604	22.9	0	0	40
N,N-dimethyl-2-(1'-methoxybutyl)aniline	16.492	4.8	$C_{13}H_{21}NO$	207	2.5	0	1	5
Dodecanoic acid (Lauric acid)	17.127	9.56	$C_{12}H_{24}O_2$	200	4.2	1	2	10
Tricyclo(4,3,1,1,(3,8) undecane 1-bromo (Adamantine derivative)	17.326	18.02	$C_{11}H_{17}Br$	228	4.5	0	0	0
1-Tetradecanol	17.468	2.9	$C_{14}H_{30}O$	214	6.2	1	1	12
3-methyl-5-ethyl-4-propyline (Amylmetacresol)	17.612	2.3	$C_{12}H_{18}O$	178	4.5	1	1	5
1-Octadecyne	17.842	2.5	$C_{18}H_{34}$	250	8.9	0	0	14
1-Dotriacontanol	19.692	2.2	$C_{32}H_{66}O$	466	16	1	1	30
Phthalic acid pentyl tridec	20.949	8.17	$C_{26}H_{38}O_4$	414	8.4	2	4	4
Cyclopropanenonanoic acid	22.165	2.23	$C_{21}H_{38}O_2$	222	8.27	0	2	15

MF- Molecular formula; MW-Mole17.612cular weight; HD-Hydrogen donar; HA-Hydrogen acceptor; RB-Rotatable bond.

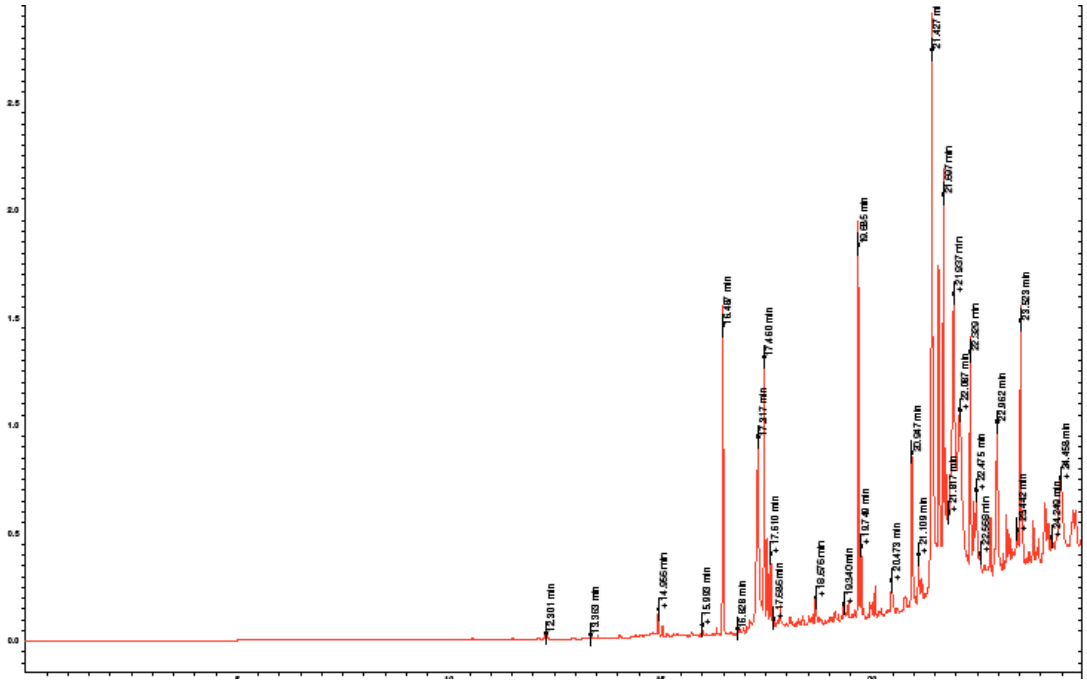


Fig. 2 GC/MS/MS Spectrum of Extracts of Endophytic Fungus, N. sphaerica

Table 3 Chemical Composition of the Extract of *N. sphaerica* (GCMSMS analysis)

Constituent	Rt	%	MF	MW	Log P	HD	HA	RB
2,5,cyclohexadiena-1,4-dione, 2-(1,1-dimethyl) (Duroquinone)	12.304	17.81	$C_{10}H_{12}O_2$	164	2.2	0	2	0
Naphthalene, 1,2,3,4, tetra	13.368	9.52	$C_{11}H_{14}$	146	3.7	0	0	0
Pentadecane	15.056	5.74	$C_{15}H_{32}$	212	7.7	0	0	12
N,N-dimethyl-2-(1'-methoxybutyl)aniline	16.489	7.83	$C_{13}H_{21}NO$	207	2.5	0	1	5
1-Tetradecanol	17.459	6.96	$C_{14}H_{30}O$	214	6.2	1	1	12
1-Octadecyne	19.684	5.70	$C_{18}H_{34}$	250	8.9	0	0	14
Phthalic acid pentyi tridec	20.472	5.67	$C_{26}H_{38}O_4$	414	8.4	2	4	4
Flabelliformin	15.994	0.31	$C_{16}H_{25}NO_2$	263	4.3	1	2	8
Benzoic acid -2(methylthio methyl ester	18.375	25.04	$C_9H_{10}O_2S$	182	2.3	0	3	3
Bifenthrin	19.060	6.23	$C_{23}H_2ClF_3O_2$	422	6	0	5	6
2-(2-cyanoethyl) 3-isopropyl 4 and 5-cyanoisoxazolidine	23.820	9.19	$C_{10}H_{15}N_{3O}$	193	0.6	1	3	1

MF- Molecular formula; MW-Molecular weight; HD-Hydrogen donar; HA-Hydrogen acceptor; RB- Rotatable bond.

Hence, the extracts of *A. flavus* and *N. sphaerica* may be considered to use as potential novel insecticides for the management of early developmental stages of teak and ailanthus defoliators, *H. purea*, *A. fabriciella* and *E. narcissus* respectively.

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