Research Article

GC-MS Analysis of Secondary Metabolites of Endophytic *Nigrospora sphaerica* isolated from *Parthenium hysterophorus*

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ABSTRACT

The present investigation involves isolation of *Nigrospora sphaerica,* as an endophytic fungus from *Parthenium hysterophorus.* The isolate has been identified on the basis of morphological and molecular characteristics. This fungus is known to produce various secondary metabolites in its culture filtrate. The FT-IR spectrum of *Nigrospora sphaerica* extract in ethyl acetate reveales the distribution of various functional groups whereas GC-MS analysis of fungal extract revealed that the polysiloxane compounds were predominant constituents. The major compounds reported in ethylacetate extract of *N. sphaerica* include Cyclohexasiloxane, dodecamethyl (21.86 %), Decamethyl Cyclopentasiloxane (19.05%), 13-Docosenamide, (Z) (15.40%), Cycloheptasiloxane, and tetradecamethyl (8.55). These compounds were not reported earlier from *N. sphaerica.*

Keywords: *Nigrospora sphaerica,* FT-IR, GC-MS, Polysiloxane, Fungal extract.

INTRODUCTION

Parthenium hysterophorus L. - a member of family Asteraceae (Tribe: Heliantheae); popularly known as congress weed, carrot weed, star weed, feverfew, white top, chatak chandani, bitter weed, ramphool, gajarghas[1,2,3], is native to the area surrounding the Gulf of Mexico, Central America, southern North America, West Indies, and Central South America. It is believed to have been introduced in India in the mid 1950's[4] as contaminant of food grains imported from the USA, but heavily introduced after 1956 through the transport of Milo (red wheat) from Mexico[5] and is now considered as one of the most feared noxious weed species spread throughout India in all kinds of disturbed habitats including roadsides, pasture lands, cultivated areas. Weeds have been proven as good sources of beneficial organisms that can potentially be utilized to protect plants from infection. These beneficial microorganisms, called endophytes, include bacteria and fungi that colonize internal plant tissues.[6] *Parthenium hysterophorus* is a much-branched, short-lived, upright, aggressive ubiquitous annual herbaceous plant which usually grows 2 to 4.5 ft. in height. Different parts of the plant are reported to be used as bitter tonic, febrifuge, emmenagogue, anti-dyscentric, etc.[7] The decoction of *P. hysterophorus* has been used in traditional medicine to treat skin diseases, wounds, ulcerated sores, facial neuralgia fever, diarrhoea, neurologic disorders, urinary tract infections, dysentery, malaria, anaemia and heart troubles [8,9,10]; fertility, fecundity and behavioral response.[11] It is also used against inflammatory skin, neural diseases and female reproductive problems.[12,13] It has been found to be pharmacologically active as analgesic in muscular rheumatism, therapeutic for neuralgia and as vermifuge.[14] *Parthenium hysterophorus* possess potential to neutralize the free radical induced oxidative damage.[15] It is the valuable source of potash, oxalic acid and high quality protein, which can be used in animal feed.[16] The methanolic extract of the flowers showed significant antitumour activity and parthenin exhibited cytotoxic properties against T cell leukaemia, HL-60 and Hela cancer cell lines.[17] Anticancer activity of *Parthenium* phenolic extract against A-498 (IC_{50} 0.5237 µg/ml) and MDA-MB231 (IC_{50} and 0.2685 µg/ml) cancerous cell lines indicated its potential to be used as anticancer agent.[18] It is reported to have insecticidal, nematicidal and herbicidal properties.[19] Phytochemical Analysis of *P. hysterophorus* revealed the presence of several secondary metabolites such as alkaloids, flavonoids, pseudoguaianolides, oils, and phenolics in all parts of the palnt. [20] The concentration of these metabolites is highest in leaves followed by inflorescence, fruit, root, and stem.[21]

The isolation of endophytic fungi from medicinal plants followed by screening of bioactive compounds has become one of the main areas of endophytic research [22, 23, 24] as medicinal plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products. Fungal endophytes colonize internal plant tissues without causing disease symptoms or apparent injury to the host.[25] These endophytes are known to produce variety of bioactive components such as alkaloids, benzopyranones, chinones, flavonoids, phenols, steroids, terpenoids, tetralones, xanthones, and others.[26] Endophytic fungi including *Alternaria helianthi*, *Alternaria alternata*, Fusarium sp. *Curvularia brachyspora*, *Cylindrocarpon* sp., Nigrospora oryzae and Penicillium funiculosum were earlier isolated from *Parthenium hysterophorus* from three sites in the central region of the state of Veracruz: Cotaxtla, Misantla and Emiliano Zapata.[27] The aim of the present study was to isolate endophytic fungi from *P.*

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hysterophorus L. and to screen their potential for the production of bioactive secondary metabolites by Gas-Chromatography Mass- Spectrophotometry (GC-MS) and Fourier Transform Infrared (FTIR) analysis.

MATERIALS AND METHODS

Sample collection

The present investigation involved the isolation of endophytic fungi from different parts of *Parthenium hysterophorous.* The plants were collected from Panjab University Campus-Sector 14, Chandigarh.

Isolation of endophytic fungi

The healthy plant of *Parthenium hysterophorous* were first washed in running water. The leaves, stems and roots were cut into pieces (5 mm in length). Surface sterilization was done in 0.01% mercuric chloride for 1min followed by washing of segments in sterilized distilled water three times.[28,29,30,31] Different plant segments were then placed on PDA medium (in Petri dishes) supplemented with antibiotic chloramphenicol (100 µg/mL concentration) to suppress the growth of bacteria. After inoculation, the petridishes were sealed with parafilm(™) and incubated at 27°C ± 10°C for 7 days. Plates were examined daily for 3-4 weeks. Fungi that grew out from sterile segments are transferred to fresh slants. The purity of culture was confirmed by transferring 4 to 5 times to PDA plates to get the pure culture[32]. Each plate was eventually examined for the purity of culture. Stock cultures were maintained by sub culturing at monthly intervals. After growing at 25°C for 7 days the slants were maintained at 15°C. From an actively growing stock culture, sub-cultures were made on fresh slants and these were used as the starting material for fermentation experiments.

Identification of fungi

Microscopic examination

The morphological identification of the fungus was done from the pure cultures. The slides were prepared in 4% KOH and stained with 2% Congo-red. Then the slides were examined with Matrix VRS-2f transmission microscope. The identification is based on colony or hyphal morphology of the fungal culture and characteristics of the reproductive structures.

Molecular Characterization

Pure fungal cultures were used to extract DNA. The DNA was used in PCR to amplify the ITS region using ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) primers described in the literature.[33] The 400 - 900 bp amplicon was gel eluted and subjected to sequencing. The sequencing results were assembled and compared with NCBI data base.

Preparation of crude extracts

The fungus was grown on PDA plates for 4-5 days at 28˚C in dark. Five discs of the isolate were cut from the actively growing culture plate and inoculated in Erlenmeyer flasks (500 ml) containing 150 ml of Potato Dextrose Broth (PDB). The flasks were incubated in BOD incubator for 21 days at 27±1°C. The fermentation broth of the endophyte was filtered through cheesecloth to remove the mycelial mats. The filtrate was extracted thrice with ethyl acetate at room temperature. The pooled extract was evaporated in a rotary vacuum evaporator.

Fourier Transform Infrared (FTIR) Spectroscopy

Fourier Transform Infrared spectroscopy was done for the detection of different functional groups present in the ethyl acetate extract of the fungus. The Fourier Transform Infrared spectra were recorded on the Thermo scientific Nicolet iS50 (FT-IR) spectrometer in range 4000-400cm^{-1}. The FT-IR method measures predominantly the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a biochemical or metabolic “fingerprint” of the sample.[34]

GC-MS analysis

The extract obtained from the above mentioned procedure was then sent for analysis by gas chromatography-mass spectroscopy (GC-MS) to CIL/ SAIF Panjab University, Chandigarh, Punjab, India. The gas chromatography- mass spectroscopy had carried out on TRACE 1300 GC, TSQ 8000 TRIPLE QUADRUPOLE MS fitted with TG 5MS (30m X 0.25mm, 0.25µm) column and S/SL Injector. The injector temperature had kept at 250˚C and MS transfer line temperature had kept at 300˚C along with ion source temperature 230˚C. The column temperature had programmed between 60˚-280˚C at 10˚C/min using helium as carrier gas at a carrier flow rate of 1ml/min. Injection volume had 1.0µl prepared in DMSO having Split flow 1ml/min. The mass spectra had taken at 75 eV with mass scan range from m/z 40-500 amu. The individual constituents had identified by comparing their mass spectra with those of standard using NIST (National Institute of Standards and Technology, U.S. Department of Commerce) compounds.

RESULTS AND DISCUSSION

Molecular characterization and identification of fungi

The colony colour of the isolate was white initially turning red later on. The conidia are borne on a hyaline vesicle at the tip of each conidiophore. Conidia are black in color, spherical to sub spherical, single-celled ranging from10-14 ×18-20 µm. On the basis of these morphological and cultural characteristics, the isolates were identified as *Nigrospora sphaerica*.[35]

To further confirm the identity, the DNA was isolated. The PCR purified product amplified at 548 bp band. The nucleotide sequences were obtained and analyzed for Basic Local Alignment Search Tool (BLAST) search

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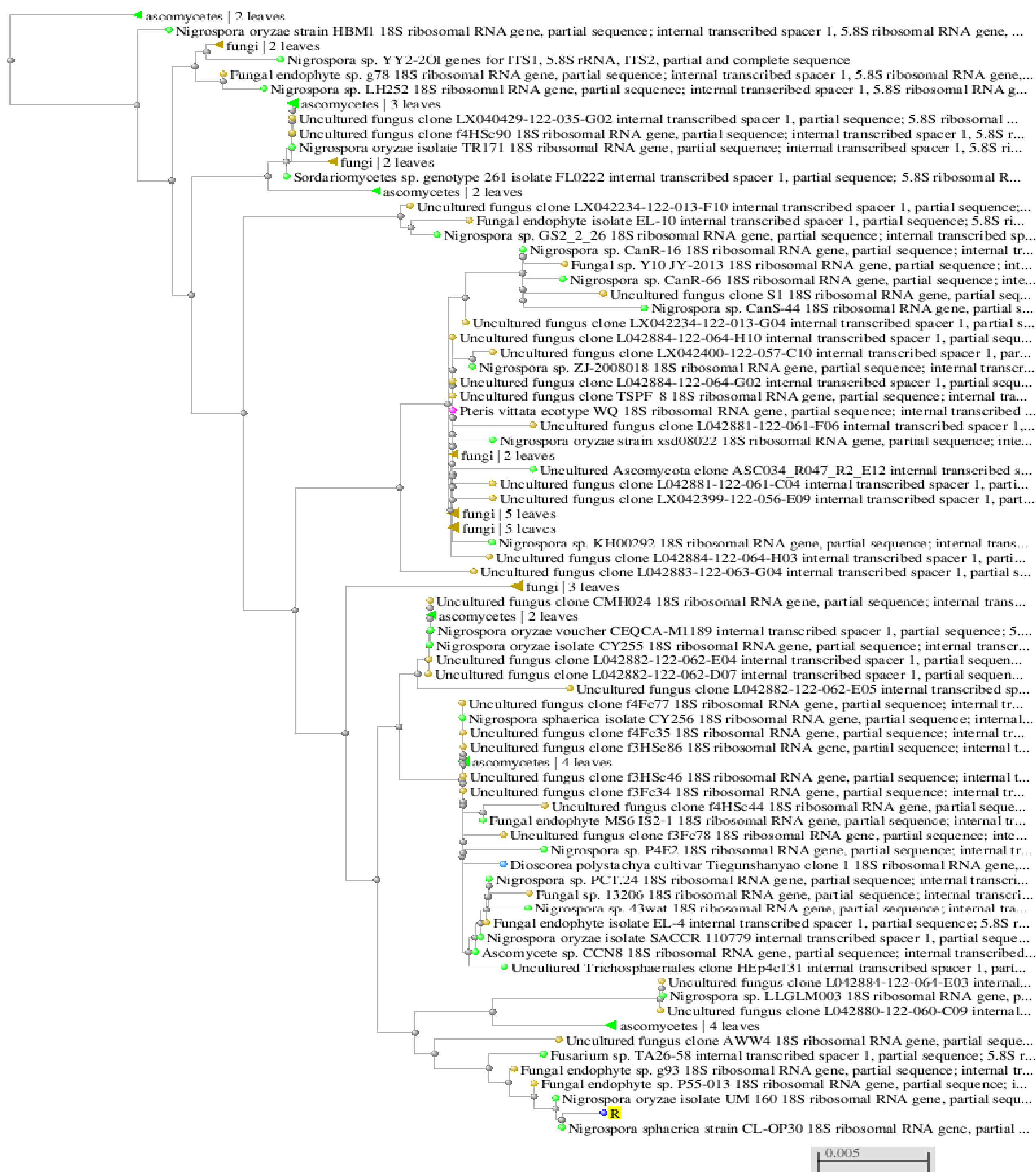
program such as (National Center for Biotechnology Information (NCBI) site) for identification. Blast search sequence similarity was found against the existing non redundant nucleotide sequence database thus, identifying the fungi as *Nigrospora sphaerica*.



FT-IR spectrum:

The FT-IR spectrum of *Nigrospora sphaerica* extract in ethyl acetate revealed the distribution of functional group within the organic fractions (Fig.1). The IR spectra were recorded in the region 4000- 400 cm^{-1}. The spectrum indicated presence of bands at 3570cm^{-1} and 3200cm^{-1} which correspond to intermolecular and intramolecular hydrogen bonding respectively. The 3052 cm^{-1} band can be assigned to alkenes (=C-H stretch) whereas the peak at 976 cm^{-1} denotes presence of germinal disubstituted alkenes. The peaks at 2929$cm^{-1}$and 2843cm^{-1} pertain to O-H stretch in carboxylic acids. The aliphatic CH stretch was observed at 2929$cm^{-1}$and 2843cm^{-1}. The peak at 2057cm^{-1}represents Carbonyl stretch. The bands at 1716 cm^{-1} and 1621 cm^{-1} can be assigned to C=O symmetry stretching while that at 1436 cm^{-1} belongs to aromatic N-O stretching and ether bonds. The 1211 $cm^{-1}$and 1072 cm^{-1} bands correspond to the S=O stretching in sulphones,

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sulphonyl chlorides, sulphates and sulphonamides. 615 cm^{-1} band is observed for halogen-substituted aromatic compounds. The band at 491 cm^{-1} represents S-S stretch (Aryl disulfides).

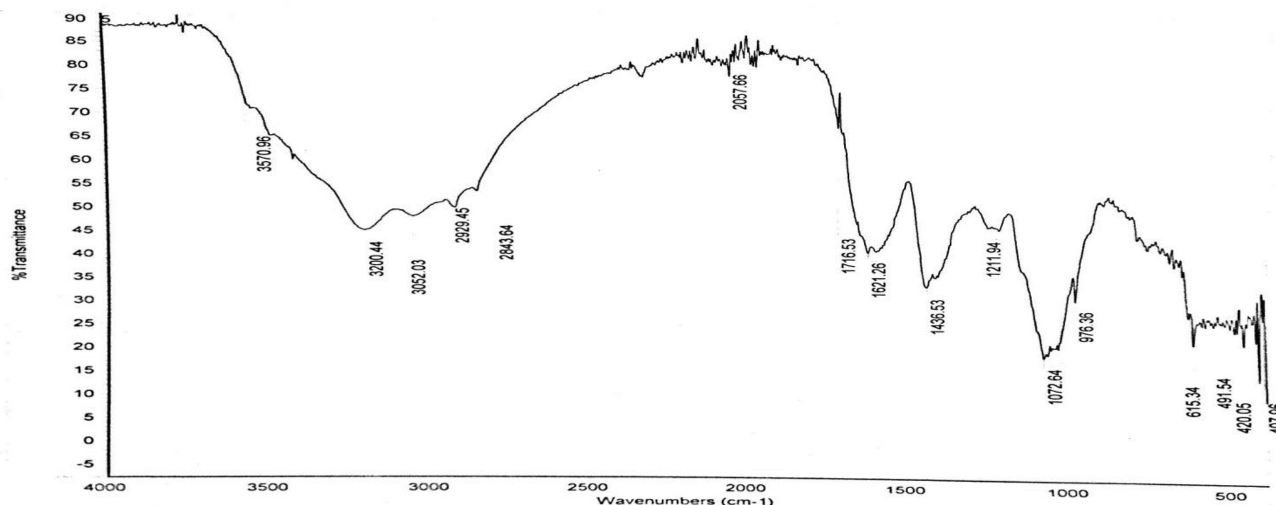

Figure 1: FT-IR spectrum of ethyl acetate extract of *Nigrospora sphaerica*

GC-MS analysis:

In this study ethyl acetate was used as extraction solvent since it is the most efficient method for obtainment of fungal secondary metabolites.[36] The ethyl acetate extract was characterized and identified by GC-MS analysis. The interpretation on mass spectrum GCMS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The active principles with their retention time (Rt), molecular formula, molecular weight and concentration percentage (area %) are represented in Fig.2 and Tables 1.

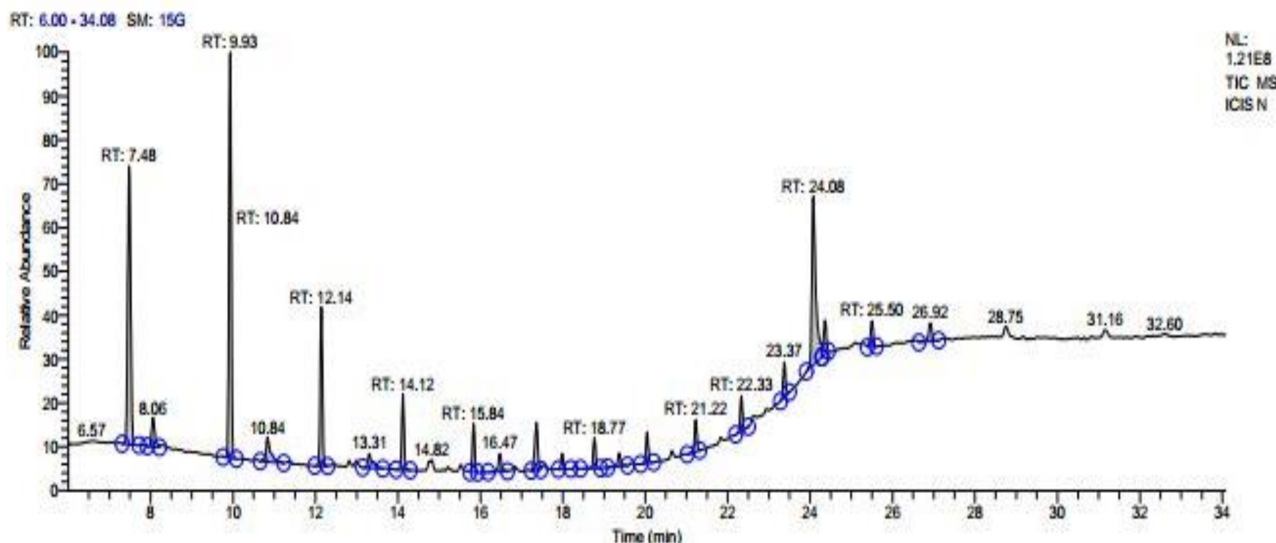

Figure 2: GC-MS chromatogram of ethyl acetate extract of *Nigrospora sphaerica*

The GC-MS analysis of fungal extract revealed that the polysiloxane compounds were predominant constituents. The major compounds reported in ethylacetate extract of *Nigrospora sphaerica* include Cyclohexasiloxane, dodecamethyl (21.86 %), Decamethyl Cyclopentasiloxane (19.05%), 13-Docosenamide,(Z) (15.40%), Cycloheptasiloxane, tetradecamethyl (8.55). Most of the identified compounds possessed many biological properties.

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Table 1: Compounds identified in the crude ethyl acetate extract of *Nigrospora sphaerica* by GC-MS analysis

S.No.	Retention Time	Area (%)	Cas #	Molecular formula	Nature of Compound	Chemical Name	Molecular Weight
1.	7.48	19.05	541-02-6	C10H30O5Si5	Cyclic methyl siloxane	Cyclopentasiloxane, decamethyl-	370
2	8.06	2.31	7206-29-3	C12H24	Alkene	6-Dodecene,(Z)-	168
3	9.93	21.86	540-97-6	C12H36O6Si6	Cyclic methyl siloxane	Cyclohexasiloxane, dodecamethyl-	444
4	10.84	2.73	64437-47-4	C16H32O	Fatty alchol	Hexadecen-1-ol, trans-9-	240
5	12.14	8.55	107-50-6	C14H42O7Si7	Cyclic methyl siloxane	Cycloheptasiloxane, tetradecamethyl-	519
6	13.31	1.57	36653-82-4	C16H34O	Fatty alchol	1-Hexadecanol	242
7	14.12	4.12	556-68-3	C16H48O8Si8	Cyclic methyl siloxane	Cyclooctasiloxane, hexadecamethyl-	593
8	15.84	2.62	556-71-8	C18H54O9Si9	Cyclic methyl siloxane	Cyclononasiloxane, octadecamethyl-	667
9	16.47	1.44	19095-24-0	C16H50O7Si8	Fatty acid ester	Octasiloxane,1,1,3,3,5,5,7,7,9,9,1 1,11,13,13,15,15-hexadecamethyl	578
10	17.36	3.46	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane, eicosamethyl-	741
11	17.99	0.88	19095-24-0	C16H50O7Si8	Fatty acid ester	Octasiloxane,1,1,3,3,5,5,7,7,9,9,1 1,11,13,13,15,15-hexadecamethyl	578
12	18.77	1.77	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane, eicosamethyl-	741
13	19.37	1.00	19095-24-0	C16H50O7Si8	Fatty acid ester	Octasiloxane,1,1,3,3,5,5,7,7,9,9,1 1,11,13,13,15,15-hexadecamethyl	578
14	20.05	1.75	556-71-8	C18H54O9Si9	Cyclic methyl siloxane	Cyclononasiloxane, octadecamethyl-	667
15	21.22	1.84	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane, eicosamethyl-	741
16	22.33	1.98	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane, eicosamethyl-	741
17	23.37	2.01	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane, eicosamethyl-;	741
18	24.08	15.40	112-84-5	C22H43NO	Unsaturated amides	13-Docosenamide,(Z)	337
19	24.37	2.29	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane,eicosamethyl-	741
20	25.50	1.74	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane, eicosamethyl-	741
21	26.92	1.60	18772-36-6	C20H60O10Si10	Cyclic methyl siloxane	Cyclodecasiloxane, eicosamethyl-	741

Cyclohexasiloxane, dodecamethyl-, also known as D_6 is found or used in the manufacture of a wide variety of products. D_6 is used in personal care products.[37] The latex of *Argemone ochroleuca* which also contain cyclohexasiloxane, dodecamethyl- (5.607%) could be a good source of antifungal agent against *Drechslera halodes* and *Candida* spp.[38]

Decamethyl- Cyclopentasiloxane also known as D_5 is an industrial chemical. It is also used in personal care products such as sunblocks, hair/skin care products, antiperspirants and deodorants.[37] Silicone polymers that may contain residual amounts of D_5 (from the manufacturing process) are used in biomedical applications and have also been approved as active and non-active ingredients in pharmaceuticals. Previous studies showed that cyclopentasiloxane decamethyl is well known antimicrobial compound isolated from different plant species [39, 40] and fungal species. [41]

13-Docosenamide, (Z) was also found in ether extract of endophytic fungus *Paecilomyces* sp. and known to posses significant antifungal and antitumor properties.[42] Antifungal activity was also exhibited by hexane extract of *Forsskaolea tenacissima* which also consisted 1.328% of 13-Docosenamide, (Z). [43] It is also used in food packaging, laundry and dishwashing products and personal care products.

Cycloheptasiloxane, tetradecamethyl is used as anticaking agent and skin conditioning agent.[37] It was also reported in ethyl acetate extract of the *Aspergillus tamarii*.[44]

CONCLUSION

The endophytic fungi produce a wide variety of bioactive compounds. Besides; being involved in the host-endophyte relationship, these compounds have potential applicability in pharmaceutical and agricultural industries. From the above study it becomes clear that *Nigrospora sphaerica* produces various secondary metabolites (most

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of the polysiloxane compounds) in its culture filtrate which are reported to have antimicrobial activity. Studies are in progress to purify these pharmaceutically important compounds. However further investigation is required to identify and elucidate the bioactive constituents which have different biological activities and may be used for human and environmental benefits.

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