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RESEARCH ARTICLE

1-Eicosane, A Hydrocarbon from *Curvularia lunata* an endophytic fungi isolated from bark tissue of *Ficus religiosa*

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ABSTRACT:

The aim of the present study was to confirm the presence of bioactive secondary metabolite, 1-Eicosane from the endophtic fungi *Curvularia lunata*isolated from the bark of the tree *Ficus religiosa. Curvularia lunata*produced1-Eicosane, an unsaturated hydrocarbon in the crude extract after three weeks of incubation in Potato Dextrose media using ethyl acetate as the organic solvent. The compound was separated by column chromatography and further analysis of the compound was done by Thin Layer Chromatography (TLC), Ultra Violet (UV), Fourier Transform- Raman (FT-R) analysis, Nuclear Magnetic Resonance (NMR) and Gas-Chromatography - Mass spectroscopy (GC-MASS).The mass spectrum of the compound showed the highest peakand the compound was identified as the straight chain hydrocarbon, 1-Eicosane, with the molecular formula $CH_3CH_{2(18)}CH_3$. The structure is further confirmed by comparison of the spectrum obtained with the mass spectrum of Eicosane from the GC-MS database WILEY and NIST respectively. Thus, the the endophytic fungi *Curvularia Lunata* serves as an alternative source for 1- Eicosane production which is used as a fuel in automobile industry.

KEYWORDS: *Ficus religiosa*, *Curvularia lunata*,Spectral analysis, Alkane, 1-Eicosane.

INTRODUCTION:

Many fungi are known to produce octane, 1-octane and other lower- molecular mass hydrocarbons[1,2,3]. Strobel *et al*., (2008) reported that *G. roseum* produced volatile hydrocarbons with antibiotic properties[4]. Several other volatile alkanes like 4-decane, 9-methyl 1-octane and 1,3-octadiene along with other esters, alcohol and fattyacids reported by them.

Other hydrocarbon derivatives such as meroterpines, dimeric naphtho-γ-pyrones, rubasperone, caryophyllene and sesquiterpenes[5,6,7,8]. Bhagobaty (2015) stated that endophytic fungi are unexplored source of volatile low molecular weight mass hydrocarbons and lipids for the production of next generation bio fuels[9].

More vigorous and focused research activity of endophytic fungi from Indian sub-continent having the potential to make fuel related hydrocarbons are in progress. Sinha *et al*., (2015) investigated *Aspergillus carbonarius* (ITEM 5010) for hydrocarbon production in liquid culture it produced several hydrocarbons like undecane, dodecane, tetra and hexadecane, o-xylene etc[10]. Strobel (2014) reported a number of endophytic fungi that could produce hydrocarbons and hydrocarbons-like volatile compounds[11].Their biological

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diversity is enormous in temperate and tropical rainforests and viewed as an outstanding source of bioactive natural products[12]. Schulz *et al*., (2002) and Strobel (2003)reported that endophytic fungi occupies literally millions of unique biological niches (higher plants) growing in plenty of unusual environments[13,14].

The study of secondary metabolism of endophytic micro-organisms developed after the finding of paclitaxel (Taxol) production by *Taxomyces andrenae,*which is theendophytic fungus associated with *Taxus brevifolia*from which paclitaxel was first isolated[15,16]. Further, plant secondary metabolites detected in endophytic fungi include naphthodianthrones hypericinfrom *Hypericum perforatum*and taxol from *Pestalotiopsis breviseta*, isolated from *Ervatamia divaricata*[17,18].

In the present study, the endophytic fungi *Curvularia lunata* isolated from the bark of the tree *Ficus religiosa*is screened for the production of hydrocarbon like compounds.

MATERIALS AND METHODS:

Isolation of the endophytic fungi:

The endophytic fungi *Curvularia lunata* VUCC 1026 (Vels University Culture Collection), was isolated from the bark tissue of *Ficus religiosa* L., growing at the Chengalpattu Reserve forest which is located at (12^0 41'N, 79^0 58' E) 50 km from south of Chennai, South India.Immediatelyafter collection, the bark segments were washedwith sterile water and the segments were surface sterilized by dipping in 70% ethanol (Merck, German) (60 Sec), immersed in 4% sodium hypochlorite (Sigma, St. Louis, MO, USA) (90 Sec) and rinsed in autoclaved double distilled water for 5 Sec[19]. The bark segments were inoculated in a Petridishcontaining PDA medium (potato-dextrose-agar)contain antibiotic streptomycin(100mg/L) to arrest the growth of bacteria and incubated in the dark at 27 ± 1^0C for three weeks.

Extraction and purification of compound:

The endophytic fungus grown in half PDA media for compound production. Fresh culture (5 day old) inoculated in liquid half PDA media for 3 weeks . After three weeks the culture filtrate passed through four-layered cheesecloth. The culture filtrate was extracted with two equal volumes of ethyl acetate and the organic phase was evaporated to dryness under reduced pressure at 35°C, which yielded a pale brown gum like material.

Characterization of compound:

Column chromatography:

The extracted compound was separated using a 1.5×30 cm column of silica gel loaded with the crude sample dissolved in hexane. Elution of the sample was done in a step by step manner using non-polar solvent mixtures of Hexane and Ethyl acetate, in the ratio, (100:0; 90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 0:100) and followed by semi polar solvent mixture of ethyl acetate and methanol in the ratio (99:1; 97:3; 95:5; 93:7; 90:10; 85:15; 80:20; 75:25; 70:30; 65:35; 60:40; 55:45; 50:50; 45:55; 40:60; 35:65; 30:70; 25:75; 15:85; 5:95; 0:100). These fractions were analyzed by Thin Layer Chromatography on silica gel under UV light after spraying with vanillin-sulfuric acid spray reagents. Similar fractions were combined and subjected to column chromatography for further purification.

Thin Layer Chromatography (TLC) analysis:

Ethyl acetate extract of *Curvularia lunata* gave 93 fractions in chromatographic separation. TLC was carried out for each fraction with a suitable mobile phase. The spots were visualized either by exposing to Iodine vapor or UV light. Some polar fractions of the silica gel chromatography showed very poor resolution of their compounds on TLC. The isolated fractions 73, showed single spot on TLC with the solvent system of chloroform/ ethyl acetate (2:1, v/v), eluted with hexane:ethyl acetate (10:90) which yielded 475 mg. The purified compound was subjected to spectroscopic data analysis.

UV Spectroscopy:

The purified sample was analyzed by UV absorption, (Shimadzu UV-2201 UV/VIS Double-beam recording spectrophotometer (1 nm resolution)).

FT-Raman Spectroscopy:

The purified sample wasfurther analyzed FT-Raman spectroscopy with a Bruker MultiRam spectrometer (Bruker Instruments Inc., Billerica, Massachusetts). This Raman system is equipped with a 1,064nm 1,000mW continuous wave (CW) diode pumped Nd:YAG laser.

Nuclear Magnetic Resonance (NMR):

The fraction 73 was characterized by proton magnetic resonance spectroscopy. The ^{1}H and ^{13}C NMR experiments were recorded using a BRUKER DRX spectrometer, which was operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C, respectively, using deutero chloroform ($CDCl_3$) as solvent, with TMS as the internal standard. MS data were measured using a low-resolution ESIMS in the positive ion mode in a MICROMASS QUATTRO-LC instrument equipped with an ESI/ APCI "Z-spray" ion source.

GC-MASS Spectroscopy:

GC-MS was performed for the fraction 73 in GC-MS-Jeol JMS GC-Mate II on a DB-5ms capillary column (30 m x 0.25 mm ID and 0.25 µm film thickness). The electron impact technique (70 eV) was used. The carrier gas was helium (99.9995% purity) at a flow rate of 1.51 ml/min, and 1 µL of the sample was injected. The injector and detector temperatures were 200°C and

180°C respectively.

RESULTS AND DISCUSSION:

Many microbial organisms are known to produce enzymes, vitamins, primary, secondary metabolites and volatiles including low molecular mass hydrocarbons[20]. Several endophytic fungi isolated from tropical host plants were investigated for lipid biodiesel production[21]. A fungal endophyte (NRRL 50072) isolated from *Eucryphia cordofolia* produces several hydrocarbons. Banerjee *et al*., (2010) isolated *Myrothecium inunduatum* from *Acalypha indica* from NE india produced many hydrocarbons and hydrocarbon derivatives[22]. The crude extract of an endophytic fungus, *Curvularia lunata* showed the presence of several compounds in complex mixtures. Characterization and purification of the complex mixture results in the isolation of major compound, 1-Eicosane.*Curvularia lunata* appears as shiny velvety-black, fluffy growth in the colony surface, having septate hyphae produced brown geniculate conidiophores (Fig.1) which are slightly curved and transversely septate.

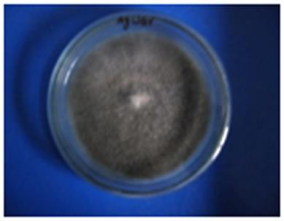

Fig.1*Curvularia lunata,* an endophyte isolated from *Ficus religiosa*

1-Eicosane in the crude extract was separated by column chromatography. Further analysis of the compound was done by Thin Layer Chromatography (TLC), Ultra Violet (UV), Fourier Transform- Raman (FT-R), Nuclear Magnetic Resonance (NMR) and GC-MASS.TLC analyses were carried out on Merck0.25-mm silica gel plates developed in the solvent systems of chloroform/ ethyl acetate (2:1, v/v) and it showed a mauve colored spot with the Rf value of 0.5 (Fig.2) which yielded 475 mg. The purified compound was subjected to spectroscopic data analysis.The spectra clearly indicate the absence of absorption at lower range particularly at UV range hence, the sample is UV-inactive and completely saturated and also indicates the absence of any characteristic chromophore (Fig.3). Probably the compound may not contain aromatic, carbonyl or conjugated aliphatic compounds and the sample could contain unsaturated aliphatic derivatives. The purified compound further subjected to FT-Raman analysis showed the existence of (sp^3hybridized carbon) C-H stretching vibration indicates- certainly there is a CH_2 or CH_3 groups. Since there is characteristic peak at 2880.50 cm-1 (Fig.4). And no other characteristic peak found in fingerprint regionit rules out the possibility of having aromatic compounds and existence of other characteristic functional groups.

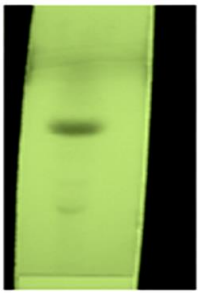

Fig.2. TLC of partially purified compound of *Curvularia lunata*

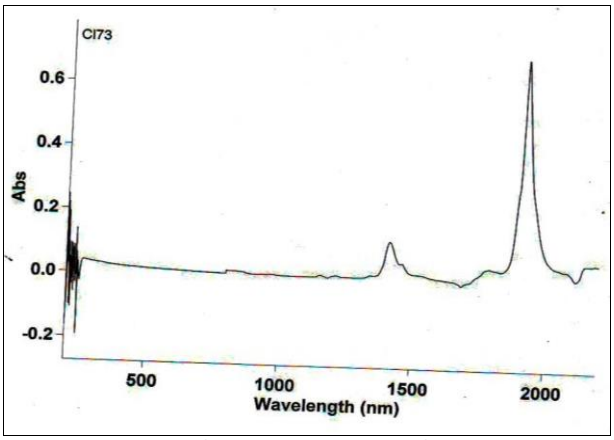

Fig.3. UV Spectrum of a compound extracted from endophyte

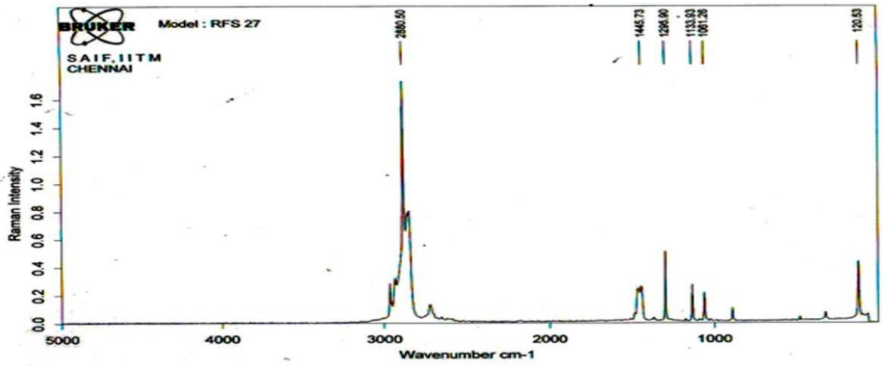

Fig. 4. FT–Raman Spectrum of a compound, 1-Eicosane

The purified compound further characterized using the ^{1}H NMR and ^{13}C NMR spectra showed several characters. ^{1}H NMR study under low resolution, the presence of two peaks. One observed in 0.8 δ and another at 1.2 δ. This was found to be a singlet and triplet in high resolution (Fig.5 & Fig.6). No other peaks are formed, hence it could be interrupted that terminal methyl (2 x CH_3) as six proton triplet (J= 6.8 Hz) at δ 0.88 and a broad methylene envelop at δ 1.26 integrating for 36 protons and $–CH_2$ at 18 times, thus showing the structure to be $CH_3CH_{2(18)}CH$.In the ^{13}C NMR the peaks are observed between 15-20 and the peak at 18.61 authentically confirm the existence of the R-CH2-R group and a triplet confirms the presence of CH_2 groups. The details for the ^{13}C NMR spectra as below:

CH_3 - CH_2 - CH_2 - CH_2 - (CH_2) - CH_2 - CH_2 - CH_2 - CH_3
14.12 - 22.72 - 29.40 - 29.70 - 29.74 - 29.70 - 29.40 - 22.72 - 14.12

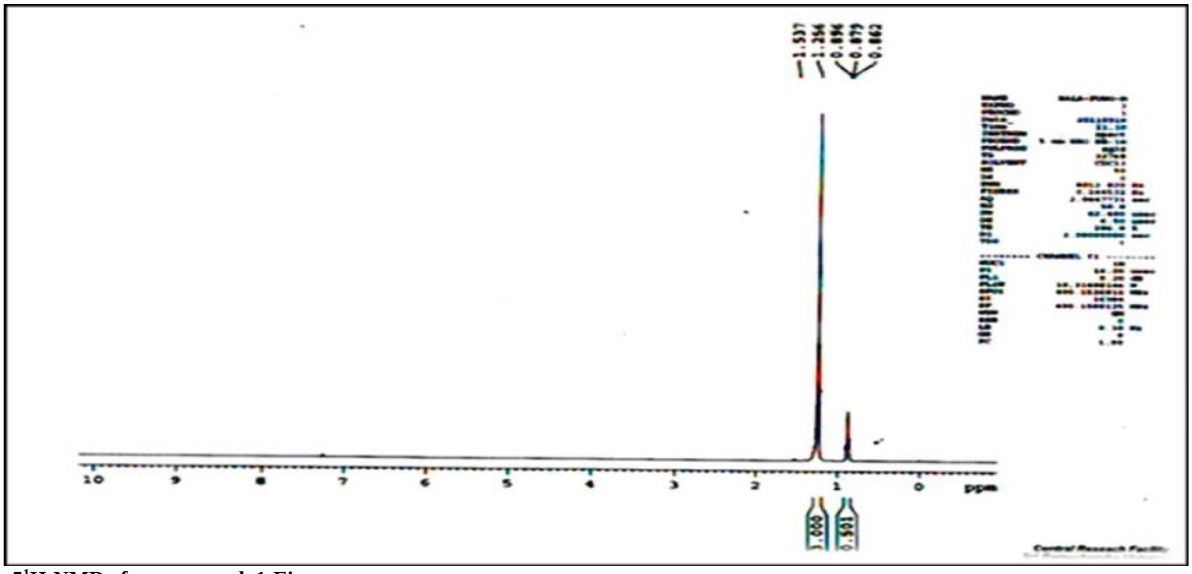

Fig. 5 ^{1}H-NMR of a compound, 1-Eicosane

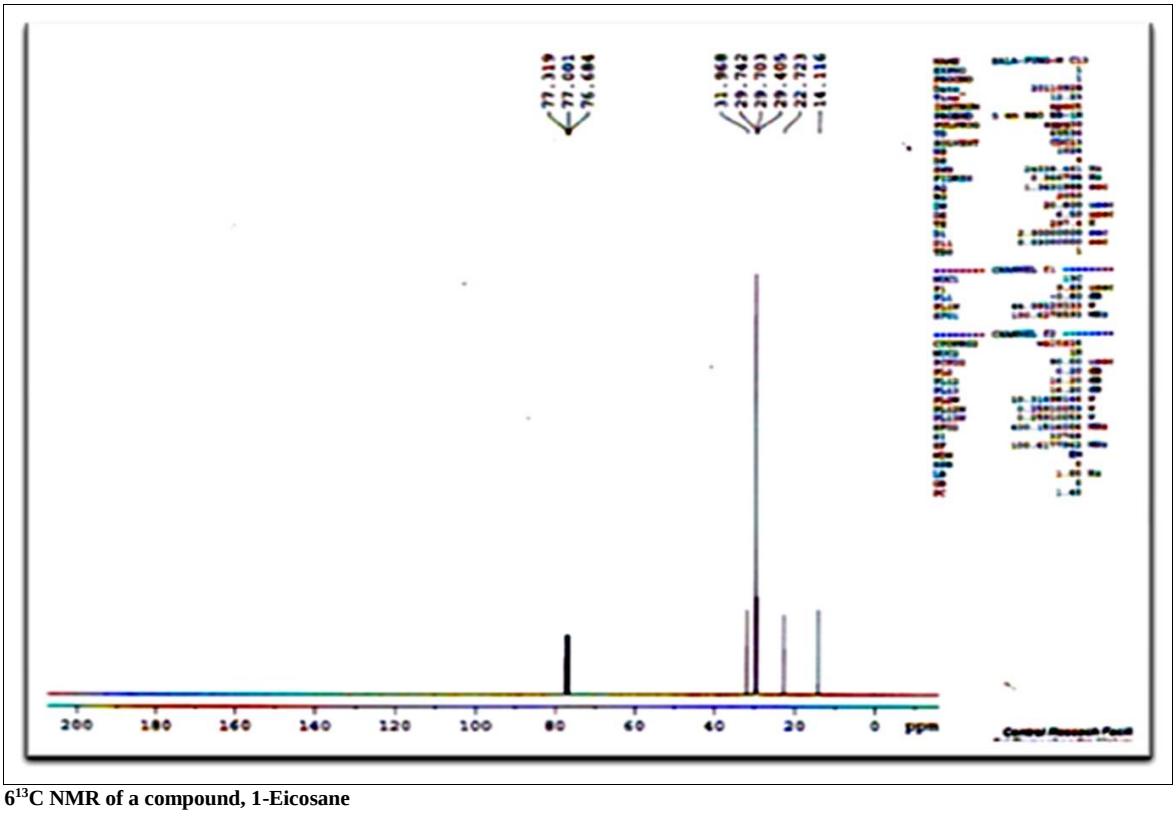

Fig. 6 ^{13}C NMR of a compound, 1-Eicosane

GC–Mass Spectroscopic analysis of 1-Eicosane show characteristic peaks at 503-506 may be due to $C_{36}+H_{36\ x\ 2+2}$ = 36 x 12 + 36 x 2+2 = 506. (Fig. 7). Subsequently, the other peaks at 355, 221 282 and 148 (Fig.4) can also be accounted in the same way as follows:-

M/e at 148 = $C_{25}+H_{52}$ 300+52 = 352
M/e at 355 = $C_{36}+H_{72+2}$ 432+74 = 506
M/e at 282 = $C_{20}+H_{4o+2}$ 240+42 = 282

Thus the fragmentation or molecular peak at 282 may be due to the presence of a compound 1-Eicosane- $C_{20}H_{20\ x2+2}$ = $C_{20\ x\ 12}$ + H_{42} = $C_{240}+H_{42}$ = 282

The mass spectrum of the compound showed the highest peak, M+ at m/z 282 and the compound was identified as the straight chain hydrocarbon, 1-Eicosane, with the molecular formula $CH_3CH_{2(18)}CH_3$. The structure is confirmed by the fragments obtained by successive loss of methylene groups (14 amu) upto m/z 43.

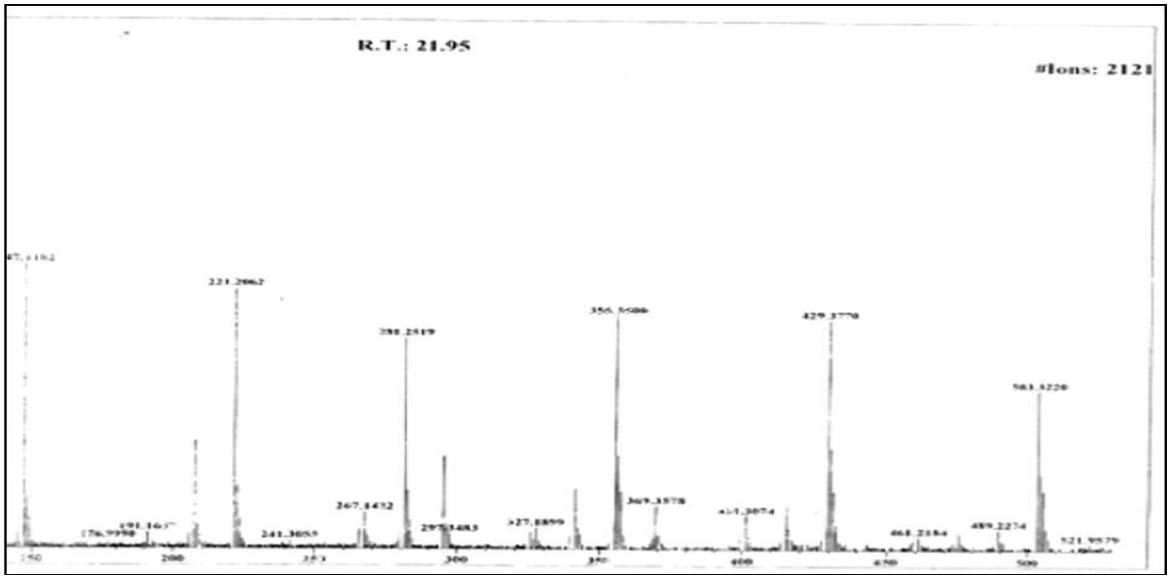

Fig. 7 GC–MASS spectrum of a compound, 1-Eicosane from 503.32 to 221.20

The structure is further confirmed by comparison of the spectrum obtained with the mass spectrum of Eicosane from the GC-MS database (Fig.7a& Fig.7b) WILEY and NIST respectively.

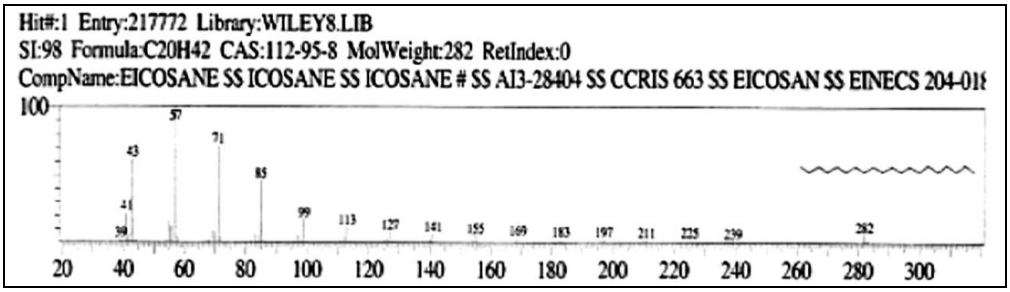

Fig.7a. GC–MASS - WILEY data base

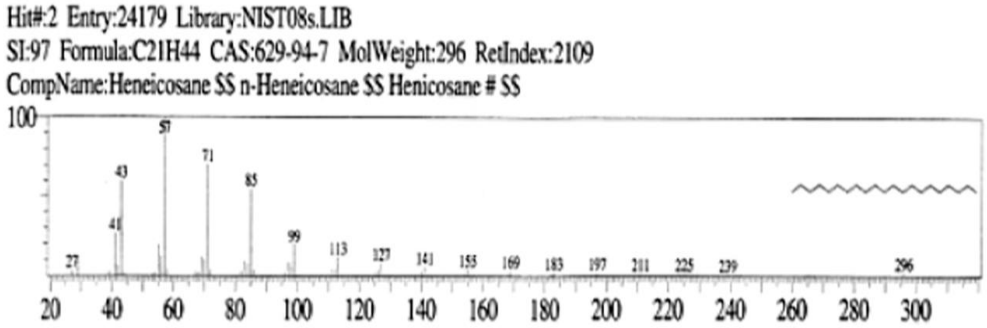

Fig. 7b. GC–MASS - NIST database

Eukaryotic and prokaryotic organisms produce hydrocarbons from fatty acids. Shall (1892) found undecane in ants and Etard (1892)found eicosane in *Bryonia dioica*[23,24]. They have usually a straight chain of with 36 carbon atoms. Sometimes they are branched with one or more methyl groups attached to it.Hydrocarbons containing odd number of carbon chains (C_{15} up to C_{33}) are mostly high in number. Several microalgae were shown to contain long-chain unsaturated alkenes from 19 to 38 carbon atoms and one to four double bonds[25]. In higher plants hydrocarbons are found at the outer surface of leaves. For example, C_{27}, C_{29}, and C_{31} n-alkanes are the most abundant (from 11 to 19%) in needle wax of the Pinaceae member, *Picea omorika*[26]. Fungi, on the other hand, commonly make long chain hydrocarbons along with a series of low-molecular mass alcohols, ethers, esters, ketones and terpenoids[2]. Wood-inhabiting fungi seem to make a wide range of volatile products[1]. The production of these volatile products by microbes is dependent upon the environmental conditions, the support medium and the microbial species being studied. Many fungi are known to produce octane, 1-octane and lower-molecular-mass hydrocarbons. The term myco-diesel was coined by (Strobel *et al*., 2008) while investigating the endophytic fungi *Gliocladium roseum* (NRRL 50072) from the stem of *Eucryphia cordifolia* since they produce the major volatile substances, including octane; 1-octane; heptane, 2-methyl; hexadecane; undecane,4-methyl; nonane, 3-methyl; and benzene, 1,3-dimethyl[27].Six methyl esters or derivatives were isolated from the extract of *Euphorbia kansui* namely, 11,13-eicosadienoic acid methyl ester ,12-octadecenoic acid methyl ester, (Z, Z)-methyl ester-9,12-Octadecadienoic acid, 10-methyl-heptadecanoic acid methyl ester, hexadecanoic acid methyl ester and methyl ester –5-Oxo-DL-Prolineis cytotoxic and induce apoptosis in SGC-7901 cells[28]. Volatile hydrocarbons from the endophyte *Fusarium solani* isolated from the bark of the plant *Taxus buccata* was active against human pathogenic microbes[29].

1-Eicosane, a C_{20} alkane is a heavy fraction alkane having the chemical formula ($CH_3(CH_2)_{18}CH_3$), molecular weight 282,55 g/mol, melting point/freezing range 35-37°C to 37-40°C, initial boiling point and boiling range 220°C at 40 hPa and Flash point > 113,00°C.1-Eicosane is the hydrocarbon, used as a PCM (Phase change material based cooling) especially as a solid-liquid PCM's for periodic power dissipating devices[30]. Other uses of 1-Eicosane in the heat storage unit placed inside the device absorbs the heat dissipated from the chips and maintains the chip temperature below the allowable service temperature of 50^{o}C for 2 hours. The hydrocarbon extracted and purified from the endophytic fungi *Curvularia lunata* from the bark of the tree *Ficus religiosa* was confirmed as 1-Eicosane, by Ultra Violet (UV) spectroscopic analysis, FT- Raman (FT-R) analysis, Nuclear Magnetic Resonance (NMR) and GC-MASS spectroscopy.

1-Eicosane is used as a PCM (Phase change material based cooling). Previous reports of antimicrobial activity of volatile hydrocarbon suggest that 1-Eicosane might also possesss the activity against pathogenic microbes and this will be the continuation of the present work in the future.Observations from the present research have important implications for fuel production with the help of a fungal resource especially from endophytic fungi and it will be a great alternative source for not only the automobile industry but also for the electronic gadgets.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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