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Research Article

Pharmacology of Rhizopus Delemer

# Phytochemical Pharmacological and Growth Promoting Study of Endophytic Fungi *Rhizopus delemer* Isolated from *Abutilon indicum* (L.) Sweet.

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**Abstract:** Endophytic fungi have been perceived as a potential source of bioactive secondary metabolites. Whereas *Rhizopus* as the endophytic fungi were isolated from the medicinal plant *Abutilon indicum* (L.) Sweet. under the family Malvaceae found in tropical areas. This work aims to isolate and identify the fungal endophyte *Rhizopus* sp. from the medicinal plant *Abutilon indicum* at the molecular level, and the second objective is to test the Plant Growth Promoting activity like Indole Acidic Acid A, Hydrogen cyanide, Phosphate solubility ($PO_4$), Catalase test, and it is the proof for plant endophyte association, lastly to analyse the extract of *Rhizopus* in phytochemical and pharmacology activity using Gas chromatography and Mass spectrometry study. The isolated fungal plant growth-promoting activity like Indole acetic acid production, Phosphate solubility assay, HCN production and Catalase enzyme synthesis was tested. As compared to IAA, $PO_4$ solubility assay, HCN and Catalase synthesis test culture gave positive results. It described endophytic fungal within-hosts that are actively responsible against pathogens and stress tolerance conditions. Isolated fungal culture was able to synthesize catalase enzymes and HCN compounds. *Rhizopus* can act as a Phyto stimulator, broad-spectrum antimicrobial agent, increased stress tolerance capacity and also can increase the water availability in the nutrition supply to the host plant. In the qualitative screening test, there are about 12 phytochemicals that showed positive results. It gives the effort of the plant to act as a defensive mechanism against pathogens. Based on morphological and phylogenetic analyses, fungal species were identified: as *Rhizopus delemer* UICC 26. Accession no: LC514308. It is an unfamiliar mutant strain. Further GC-MS analysis revealed that the Identification of 20 bioactive compounds in the ethanolic extract of fungus *Rhizopus delemer* was Butane, 1,1-diethoxy-2-methyl-. 3,3-diethoxy-2-butanone. identified. The identification of bioactive chemical compounds is based on a Database in the National Institute of Standard Technology. The compound's retention time, peak area, molecular weight and molecular formula were matched with WILEY and NIST Library. It could be concluded that the isolated endophytic fungus Rhizopus delemer UICC 26 extract was prominent endophytic fungi in *Abutilon indicum* plant and also it enhances the plant growth-promoting activity. It was confirmed by the positive results of HCN and Catalase production test. In 12 different phytochemical compounds, and among 20 bioactive agent's presence 9 shown our fungi has active form inside of plant part against the pathogen.

**Keywords:** *Endophyte fungus, Abutilon indicum, Rhizopus delemer, GC-MS, HCN and Catalase enzyme.*

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## 1. INTRODUCTION

Endophytes are living inside the cells without causing any infection or morphological changes in the outer appearance of the host[1]. An endophyte is an endosymbiotic, often a bacterium or fungus that enhances host growth, through nutrient uptake levels and improves the plant's ability to tolerate environmental conditions[2]. Many ongoing research and studies were overlapping in the plant-fungal relationship. Fungal mycelium has an intense ability to produce enormous active metabolites and useful compounds when it is inside the plant system. The *in-vitro* fungal culture extract has the same bio compound that resembles its host. Among various fungal endophytes, *Rhizopus* Sps., is a rhizosphere biome habitat. It comes under the Mucaraceae family. There are about 7 to 8 well-known *Rhizopus Sp.,* around in plant and soil environments. *Rhizopus* is a saprophytic to endosymbiotic nature[3]. In a similar point of endosymbiotic association of *Rhizopus Sps.,* harbour its cytosol part with bacterial cell, especially bacteria *Burkholderia Sp*[4,5]. In this fungal-bacteria relationship, rice blight disease-causing agent rhizoxin is a phytotoxin in seedlings of rice, and it is an antitumor drug in eukaryotes[6]. Artursson et al report says bacteria-fungal mutualism increases the level of sporulation[7] in seedling of rice. In the same order, Iwasaki S et al reports says *Rhizopus* as endosymbiont leads to the plant's capacity to function as pest control[8]. These findings were confirmed by complete genome sequence[9],16S rDNA phylogenetic tree analysis[10]. Almost all plants are known to harbour endophytes. Endophytic fungi have a source of powerful bioactive agents[11]. In GC-MS analysis secondary metabolites Methyl eugenol compound were food additive, antifungal and insect attractant[12]. It was isolated from endophytic fungi *Rhizopus sps,* it is from the plant of Apopcynaceace. Methyl eugenol compound inhibits the colonies of pathogen *Aspergillus flavus*. In which *Rhizopus* fungal isolated from Peanut and Kernal plant[13]. The endophytic fungi of *Rhizopus* were isolated from Foliar plant leaves of *Debregrasia salicifolia*. It has high value of antioxidant and the high-value zone of inhibition in antibacterial activity, especially in *E. coli* strain. Here the identified strain name *Rhizopus* DL2H[14]. Another report that the fungal extract of *Rhizopus Sps.,* isolated from *Crocus sativus*. The evaluation of extract says high efficiency in antioxidant and antibacterial activity[15].In this analysis, important compound of alpha and beta iron isolated from *Rhizopus sps*, this production is similar to host plant[16] *Achilea milefolium* plant. In addition, the author's review says that *Rhizopus* is an endophyte in many of the plants species, which enhances the host growth. But very rare area of researches has undergone the molecular characterization of *Rhizopus sps* for identification. Among identified *Rhizopus,* some of them underwent the screening of bioactive compound extraction using GC-MS study. For example, in Endophytic fungi, *Aspergillus clavatonanicus* strain MJ31, isolated from the root system of *Mirabalis jalaba* plant and analysed their 28 volatile secondary metabolites in GC-MS study[17]. Mohammed et al reported that, the healthy leaf midrib portion of *Tectona grandis* tropical tree, isolated the endophytic fungi *Phomopsis* Species*,* its Secondary metabolite analysis especially in insecticidal activity[18]. Endophytic fungi *Chaetomium globosum* isolated from seed samples of *Moringa olifera* plant and undergone to its secondary metabolite analysis[19]. Here also our primary aim of the work is to isolate and identify the fungal endophyte *Rhizopus* Sp., from the medicinal plant *Abutilon indicum,* in molecular level. The second objective is to test the Plant Growth Promoting activity like IAA, HCN, Phosphate solubility, Catalase test. It is the proof for plant endophyte association, finally to analyse the extract of *Rhizopus* in phytochemical and pharmacology activity using Gas chromatography and Mass spectrometry study.

## 2. MATERIALS AND METHODS

### *2.1 Collection of Plant Sample*

The *Abutilon indicum* plant leaves were collected from Gandhi Grammam South Karur, Tamil Nadu, India. The plant was identified as Tutti, authenticated the specimen were matched with Herbarium flora of Tamil Nadu, Carnatic accessed number 2960 by Mathew, 1960[20] and confirmed by, the Director, Rapinat Herbarium, St. Joseph's College, Tiruchirappalli, Tamil Nadu India. A total of 50 leaflets were carefully selected from shrub and were brought to the laboratory for the Isolation of fungal endophyte.

### *2.2 Isolation of Fungal Endophyte*

Selected leaves were washed with running tap water to remove leaf debris and unwanted particles. The explant leaves were immersed in ethanol (70%) for 20 seconds dipped in (0.52%) Sodium Hypochlorite for 30 seconds then washed with sterile water for 3 times at regular intervals and then air-dried the sample in tissue paper, make this explant leaves portion into bore 6mm using one sterile whole puncher and transferred into PDA plates with supplemented Streptomycin (250 mg/ltr) to avoid bacterial growth. Incubated the plates at room temperature for 2 weeks, and observed the fungal growth at 3-4 days. Emerging mycelia from the end of explant were subculture in freshly prepared PDA plates (Water 1000ml, potato (sliced washed and peeled) 300g, glucose 20g, and agar 20 g) and obtained pure cultures[21,22]. Stock cultures were maintained in PDA slants and stored at 4°C. Further, the analysis of bioactive agents in fungal isolates was then inoculated to a rotary shaker system at 25°C for three days.

### *2.3 Morphological Identification of Fungal Endophyte*

Fungal morphology was identified using the Lactophenol cotton blue (LPCB) staining method. Placed a drop of 70% Alcohol on a microscope slide. Immersed the fungal culture into a drop of alcohol. Add one or two drops of LPCB Stain before the alcohol dries out. Observed the slide under a trinocular microscope at 100x magnification[23].

### *2.4 Fungal DNA Isolation*

Isolation of fungal DNA is done by the Standard salting out procedure. 0.24 g of agarose powder was soaked in 30 ml of 1x TAE buffer and boiled until it formed a clear solution. At approximately 50°C it was allowed to cool down. Then added 1.5 µl of ethidium bromide and mixed well in the solution. Kept at room temperature for ½ hours for solidification as it was poured into gel casting plate with an already adjusted gel comb. The gel was soaked in 1X TAE buffer in the electrophoresis tank. 3µl of DNA with 3µl of gel loading dye was loaded in the wells using micropipettes. It was run at 70 V for 15 to 20 min[24,25]. Orange colour DNA bands were observed in the UV transilluminator.

### *2.5 PCR Amplification*

PCR Thermal cycler (Gene AMP PCR 9700, Applied Biosystem, USA). Big Dye Terminator v 3.1 cycle sequencing kit on ABI 3730xl genetic analyser was used. ITS1 and ITS 4 Primer approximately taken in ~700bp of ITs region, further

undergoing to aligner software Mega 7. More than 100 bases aligned in blast n and used clustal W analysis for multiple sequence alignment[24,25].

### 2.6 *Phylogenetic Tree Analysis*

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The analysis involved 11 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3r^{d}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 568 positions in the final dataset. Evolutionary analyses were conducted in MEGA7[26].

### 2.7 *Phytochemical Screening*

Endophytic fungal isolates were further inoculated into 250 ml Erlenmeyer flasks containing 100 ml Potato Dextrose Broth and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The broth culture was filtered to separate the mycelia and filtrate. After that, equal volume of ethyl acetate was added to the filtrate. it was mixed well for 10 min and kept for 5 min till the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using a separating funnel. The extract was concentrated by removing the solvents under reduced pressure at 35-40°C with rotary evaporation. The extract was dissolved in DMSO and stored at 4°C[27].

### 2.8 *GC-MS Study*

The fungal extract was subjected to GC MS analysis to identify the bioactive compound. GC-MS QP2010 Plus (Shimadzu, Kyoto, Japan) system was utilized. The system was equipped with an auto injector (AOC-20i), headspace sampler (AOC-20s), a mass selective detector with an iron source (220 °C) and an interface (260 °C). Rtx-5 MS capillary column having 30 mm X 0.25 mm of length X diameter and 0.25 µm of film thickness was used for MS analyses. The mass range of 40-650 m/z with 1,000 ev of the threshold was proposed. The injector was set in the split injection mode having 250 °C of temperature. The ratio applied for split mode was 10.0. The starting temperature was adjusted to 80 °C (3 min), which afterwards increased to 280 °C with a ramp rate of 10 °C/min. Helium (>99.99 %) with 40.5 cm/s of linear velocity was employed as a carrier gas. The system was programmed with 16.3 ml/min of total flow rate and 1.21 ml/min of column flow. Components were recognized by their retention time (RT) and elucidation of mass spectra. The spectral fragmentation of unknown components was compared with the known and standard components provided by the databases of WILEY&NIST[28].

### 2.9 *Plant Growth Promoting Activity*

#### 2.9.1 *IAA Test*

The test microorganism was inoculated in tryptone broth and incubated for 24h. After incubation, the culture was allowed to centrifuge at 10,000 rpm for 15 min. 2mL of supernatant was collected and it was added with 2 drops of orthophosphoric acid and 4mL of Salkowski reagent. The solution was incubated for 25 min at room temperature[29,30].

#### 2.9.2 *Phosphate Solubilization assay*

0.5mL of culture was added with 0.4mL of 10N sulphuric acid and 0.8mL of 2.5% ammonium molybdate. Later, 0.4mL of amino naphthol was added to the mixture and incubated for 15-20 min at room temperature. The absorbance was read at 570nm[29,30].

#### 2.9.3 *Hydrogen cyanide test*

The nutrient agar medium was prepared using 0.44g of glycine for 100mL and plated in a Petri dish. The microorganism was spread over the plate containing the medium. Whatman No.1 filter paper was soaked in 50mL of 2% $Na_2CO_3$ and 0.5% picric acid solution. The filter paper was later placed on the medium and the petri dish was sealed using parafilm. The plate was incubated for 4 days at room temperature[29].

#### 2.9.4 *Catalase test*

The fungal culture was placed on the cavity slide and it was air dried. Few drops of 3% $H_2O_2$ solution were added to the slide[29].

## 3. RESULT

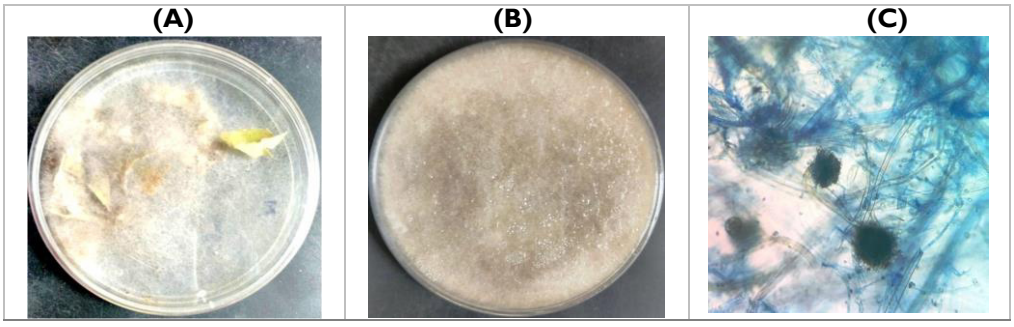


**Figure I: Isolation of Endophytic fungi *Rhizopus delemer* A) Emerging mycelium from leaf tip B) Pure culture C) Sporangia with spores**

Figure I shows the isolation of endophytic fungi *Rhizopus delemer* pure culture plates. Here, Figure A shows the plates which contain the growth of fungi from (*Abutilon indicum*) plant leaf pieces in Potato dextrose agar plates, Figure B represents isolated pure culture plates of fungi *Rhizopus* figure I C shows Electron microscopic image of the mycelium growth of fungi *Rhizopus*.

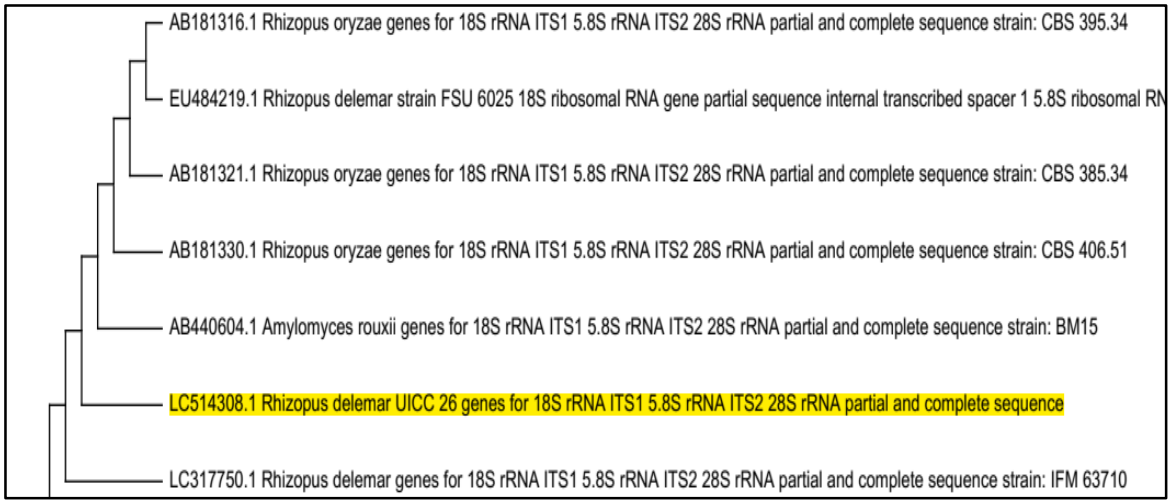


**Fig II: Phylogenetic tree of *Rhizopus delemer***

Figure II explains the Phylogenetic tree of *Rhizopus delemer* fungi LC514308. Here Phylogenetic tree confirms the species identification of isolated endophytic fungi from *Abutilon indicum* plant. Among various *Rhizopus* species, our blast search results shown 98% *Rhizopus delemer*. This is the unfamiliar mutant fungal strain. Whereas the result of LC514308.1 *Rhizopus delemer* fungi UICC 26 gene was identified and named of fungi from tree results, Ribosomal subunit was internal terminus sequence 1 - 5.8 S rRNA subunit and internal terminus sequence 2 - 28 S rRNA partial and complete sequences.

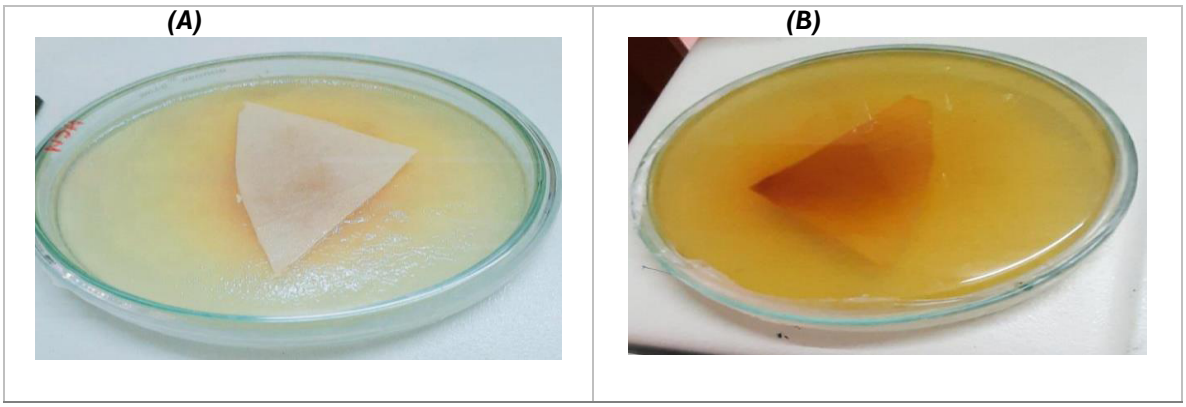


**Fig III: HCN Production test A) Before incubation B) After incubation**

Figure III Shows plant growth enhancement test of Hydrogen cyanide production for endophyte *Rhizopus delemer* fungi. Here, figure A shows the before incubation of the nutrient agar plate. Figure B shows the orange colour formation of Hydrogen cyanide production by our culture *Rhizopus delemer* fungi. From these results, fungal culture has biocontrol activity against pathogens.

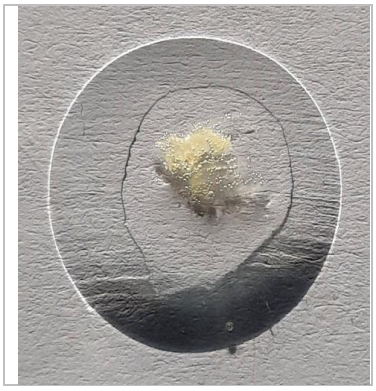

**Fig IV: Catalase enzyme test**

Figure IV: shows plant growth enhancement test of Catalase enzyme production for endophyte *Rhizopus delemer* fungi. Here figure IV explains the oxygen bubble formation of fungal culture using hydrogen peroxide. It shows fungal *Rhizopus delemer* has lethal activity against pathogens. When it is in upstream processing this Plant growth-promoting test used to increase the production of Catalase enzyme in *in-vitro*.

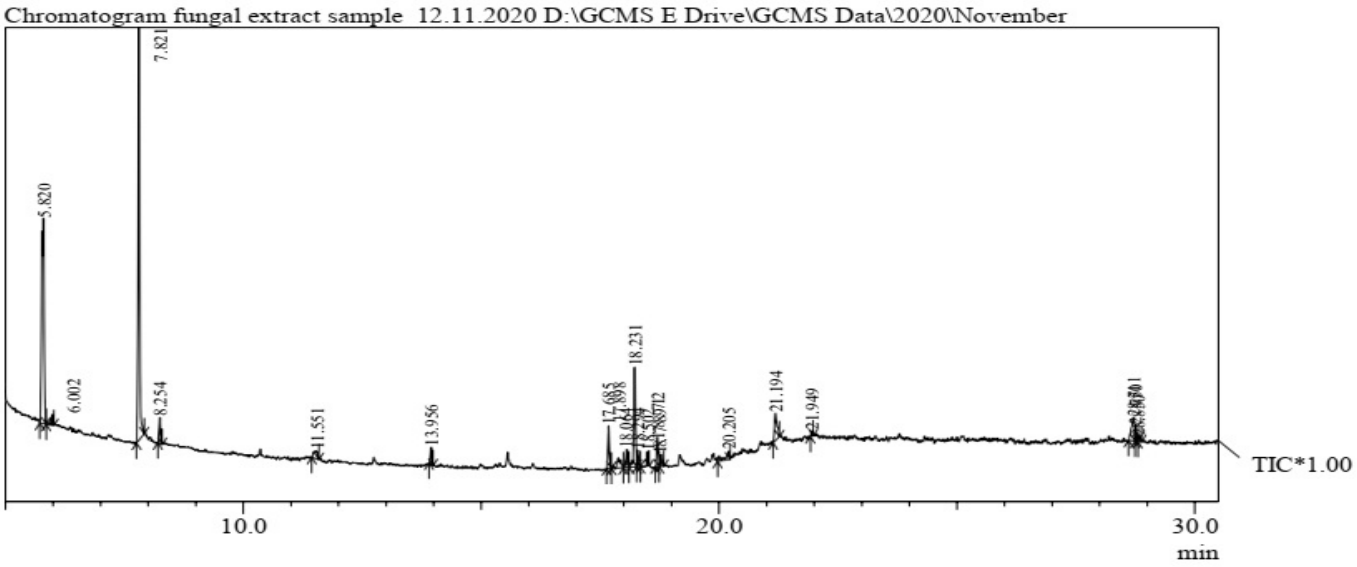


**Figure V: GC-MS Study of *Rhizopus delemer* fungal Ethanolic extract chromatogram**

***Figure V: Explains GC-MS of Rhizopus delemer fungal extract. It gives bioactive agents in various peaks with the retention time***

| **Table I: Morphological identification of *Rhizopus delemer* in Trinocular microscope** | | |
|---|---|---|
| **Microscopic description** | **Observation** | **Organism** |
| Colour | Brownish grey to blackish grey | |
| Zonation | Regular | |
| Sporangiospores | Angular, Sub globose to ellipsoidal, With Striation | |
| Sporangia | Globose, flattened Base, greyish Black, powdery in appearance | *Rhizopus Spp.* |
| Height | 5-8mm | |
| Columella | Globose, sub globose or oval, umbrella-like form | |
| Spore | Present | |

Table I explains the morphological identification of *Rhizopus delemer* in Trinocular microscope. Which was identified by various forms of morphological description Colour, Zonation, Sporangiospores, Sporangia, Height, Columella, Spores. The observation of Culture colour was brownish grey to blackish grey, Zonation was regular, Sporangiospores were angular, subglobose to ellipsoidal, with striation, Sporangia was globose, flattened Base, greyish Black, powdery in appearance, Height of culture 5-8mm, columella was Globose, subglobose or oval, umbrella-like form, Spores were present. These observations confirmed the isolated fungi were *Rhizopus* sps.

| ***Table II: Plant growth promoting properties of Rhizopus delemer fungi*** | | |
|---|---|---|
| *Name of test* | ***Observation*** | ***Result*** |
| *IAA* | No colour | Negative |
| *$Po_4$ Solubility* | No colour | Negative |
| *HCN* | Orange | Positive |
| *Catalase test* | Oxygen bubble | Positive |

Table II: shows the enhancement of plant growth by endophytic fungi *Rhizopus delemer*. Here, HCN test and Catalase test have given positive results. Which was confirmed by the observation of plates. The plates were incubated with What man no:1 Filter paper. when it changes into colour less to orange colour formation, which was positive result for HCN test. The testing culture of *Rhizopus delemer* was producing oxygen bubbles and water while pouring Hydrogen peroxide which was positive result for catalase enzyme test.

| **Table III Primary Qualitative Phytochemical screening in ethanolic extract of *Rhizopus delemer*** | | |
|---|---|---|
| *Phytochemical Constituents* | *Observation* | *Fungal extract* |
| *Tannins* | Green | +++ |
| *Saponins* | Froth form | +++ |
| *Steroids* | Reddish brown | + |
| *Terpenoids* | Reddish brown | ++ |

| | | |
|---|---|---|
| *Cardiac glycosides* | Violet or brown | +++ |
| *Anthraquinones* | Pink, violet or red colour | + |
| *Glycosides* | Violet to blue to red colour | + |
| *Phenols* | Reddish orange | +++ |
| *Alkaloids* | Yellow | +++ |
| *Xanthoproteins* | Blue-black | +++ |
| *Emodin* | Red | + |
| *Carbohydrates* | Reddish violet | + |

***(Slightly present=+, Moderately present=++, Strongly present=+++)***

Table III shows the important 12 phytochemical compounds which were present in the Endophytic fungi *Rhizopus delemer*. Whereas Tannins, saponins, Cardiac glycosides, Phenols, Alkaloids, Xanthoproteins are strongly present and also Terpenoids were moderately occur, lastly Steroids, Anthroquinones, Glycosides, Emodin, Carbohydrates were slightly present in the extract of *Rhizopus delemer* fungi.

**Table IV Bioactive Chemicals Identified in *Rhizopus delemer* fungal extract**

| R.Time | Area% | Height% | Compound name | Molecular formula | Molecular weight |
|---|---|---|---|---|---|
| 5.820 | 27.94 | 20.38 | butane, 1,1-diethoxy-2-methyl | $C_9H_{20}O_2$ | 160 |
| 6.002 | 1.01 | 1.05 | 3,3-diethoxy-2-butanone | $C_8H_{16}O_3$ | 160 |
| 7.821 | 33.20 | 40.96 | propane, 1,1,3-triethoxy | $C_9H_{20}O_3$ | 176 |
| 8.254 | 1.95 | 2.55 | 1,1,3-triethoxybutane | $C_{10}H_{22}O_3$ | 190 |
| 11.551 | 1.50 | 0.78 | 1,3-dioxolane, 2-(phenylmethyl) | $C_{10}H_{12}O_2$ | 164 |
| 13.956 | 1.17 | 1.69 | 3-ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (tri) | $C_{17}H_{50}O_7Si_7$ | 562 |
| 17.685 | 3.29 | 4.22 | 1,3-diphenyl-1,3,5,5-tetramethyl | $C_{16}H_{22}O_3Si_3$ | 346 |
| 17.898 | 1.91 | 1.01 | 2h-pyran-2-carboxaldehyde, 3,4-d | $C_8H_{12}O_2$ | 140 |
| 18.064 | 1.74 | 1.79 | alpha. -d-mannopyranoside, methyl 3,6-anhyd | $C_7H_{12}O_5$ | 176 |
| 18.231 | 8.81 | 9.87 | 3,7,11,15-tetramethyl-2-hexadecen-1-ol | $C_{20}H_{40}O$ | 296 |
| 18.294 | 1.40 | 1.64 | 1-decene, 8-methyl | $C_{11}H_{22}$ | 154 |
| 18.502 | 0.89 | 1.41 | Bicyclol [4.1.0] heptane, 3-methyl | $C_8H_{14}$ | 110 |
| 18.712 | 2.60 | 2.96 | 1-hexadecyne | $C_{16}H_{30}$ | 222 |
| 18.789 | 1.08 | 1.23 | silane, diethyl(1-phenylpropoxy) | $C_{22}H_{32}O_2Si$ | 356 |
| 20.205 | 1.62 | 0.82 | phosphorodiamidic acid, tetramethyl-, pen tach | $C_8H_{14}O_2$ | 142 |
| 21.194 | 3.85 | 2.67 | oxirane, decyl | $C_{12}H_{24}O$ | 184 |
| 21.949 | 0.31 | 0.56 | 1,4-cyclohexadien, 1,3,6-tris (trim) | $C_{15}H_{32}Si_3$ | 296 |
| 28.711 | 3.98 | 2.36 | 1,2-benzenedicarboxylic acid, diol | $C_{24}H_{38}O_4$ | 390 |
| 28.750 | 1.16 | 1.60 | 2-methoxy-1-methylpropyl acetal | $C_7H_{14}O_3$ | 146 |
| 28.850 | 0.59 | 0.46 | (15)-n-labelled -dimethylamine | $C_4H_{11}N$ | 73 |
| | 100% | 100% | | | |

Table IV explains 20 various bioactive compound production using Gas chromatography and Mass spectroscopy**.** Table IV explains 20 various bioactive compound production using Gas chromatography and Mass spectroscopy**.** Here maximum percentage area was found for butane, 1,1-diethoxy-2-methyl (27%) and propane, 1,1,3-triethoxy (33%). Higher molecular weight (562) was found for the compound 3-ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (tri.) at a retention time of 13.956. Compound (15)-n-labelled -dimethylamine had very low molecular wt (73) which was obtained at a retention time of 28.850.

**Table V Pharmacological activity of bioactive compound**

| Phytoconstituents | Biological activity |
|---|---|
| 3,7,11,15-tetramethyl-2-hexadecen-1-ol | Antimicrobial, Antioxidant, Anti-inflammatory activity. |
| 1,2-benzenedicarboxylic acid, dio | Used as a plasticizer for vinyl foams which are often used as floor tiles. Other uses are in traffic cones, food conveyor belts, and artificial leather. |
| 1,1,3-triethoxybutane | Anti-nephrotoxic Activity, Hydrophobic modifiers in Wastewater treatment. |
| 3-ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris | Antifungal, Antibacterial, Antiviral Activity. |
| silane, diethyl di(1-phenylpropoxy) | Anticorrosion agent, Adhesion promoters, Crosslinking agents, Water scavengers, Coupling Agents. |
| 2-methoxy-1-methylpropyl aceta | Antioxidant, Antibacterial, Anticancer, Anti photoaging activity. |
| (15)-n-labelled -diethylamine | Anticancer activity. |
| 1-hexadecyne | Stabilizing the nano compound in drug delivery. |
| alpha.-d-mannopyranoside, methyl 3,6-anhyd | Precursor of Synthesising the oligosaccharide. |

Table V Pharmacological activity of bioactive compound in *Rhizopus delemer* fungi. Table V explains among 20 bioactive compounds; 9 compounds have active form. The isolated compounds have various application oriented. There are Encapsulate in nanoparticles, anticancer, antifungal, antibacterial, antioxidant, antiviral, anti-inflammatory and precursor for oligosaccharide compound synthesis.

## 4. DISCUSSION

### *4.1 Isolation and Molecular Identification of Rhizopus delemer*

From the second to third day, observations were for the growth of the most protruding fungi over the study. Figure (Figures, I A, B) represents most persistent fungus and was transferred to a fresh PDA plate by mycelial tipping and sub-cultured with the media-containing plates which was amended with Streptomycin under sterile conditions. The plates were incubated at 27 °C for about 3-4 days. In Table (Table I) explains the Micro and macroscopic appearance of the fungal colonies, the shape, appearance and the function of spore production and characterization of the spores was observed using the standard mycological manuals[30]. The Identification of fungi was based on the shape, method of production of spores and its arrangement (conidial ontogeny). In Figure (Figure I C) shows that under Trinocular Microscope, the following wet mount preparation by using lactophenol cotton blue staining. The microscopic slides were observed under 10X, 40X and 100X objective lens. The identified fungi were found to be *Rhizopus Sps*., which belongs to the Mucaraceae family. Again, this Table (Table I) explains the Zonation of mycelium growth regular, filiform, hyphae filaments are non-septate. The colour of mycelium was white, and it becomes brownish-grey to blackish grey when mature. Spores were present, Height of the moulds 5-8 mm, Columella become globose, subglobose or oval, umbrella-like form. Sporangia are in form of the globose, flattened base, greyish black, powdery in appearance. Sporangiospores are angular, subglobose to ellipsoidal, with striation according to *Rhizopus delemer* (Wehmer and Hanzawa 1912). In BLAST search for the fungus isolates *Rhizopus delemer* revealed 98% similarity to *Rhizopus delemer* strain UICC 26. GenBank accession Number: LC514308. Figure (Figure II) shows the results of phylogenetic tree *Rhizopus delemer* fungal isolates.

### *4.2 Plant Growth Promoting Properties*

#### *42.1 HCN Production*

HCN test is mainly used to analyse the catabolic and biocontrol ability of culture. Figure and Table (Figure III A, B and Table II) show the results of our *Rhizopus delemer* fungal culture in Hydrogen cyanide test. It was confirmed by positive after Incubation time period with What man no:1 filter paper that was soaked in 50 mL of 2% $Na_2CO_3$ and 0.5% picric acid solution mixture. *Rhizopus delemer* fungal has the ability of HCN compound-producing activity. It is a broad-spectrum antimicrobial character[31], phytostimulator[32], stress tolerance factor [33] within host, and Tomaž et al reports says HCN as a meta complexing agent and weathering in Geochemical process regulation of $N_2$, $S_2$, C, Phosphate cycle[34] and increases the availability of the nutrients in Tomato plants influenced by endophytic fungal culture[35, 36]. Here Glycine is a precursor for HCN production[37]. HCN synthase encoding genes are hcn bc. It has the biocontrol role in Fluorescent *Pseudomonas sps*[38]. Rhizosphere biome organisms are mostly able to act as an antifungal agent [39], against to weeds seedling growth[40] and also against to plant disease-causing pathogens[41]. These values are because of the production of HCN by endophytic fungi *Rhizopus delemer.*

#### *4.2.2 Catalase enzyme activity*

A major act of catalase enzyme in an organism is an Electrocatalyst[42]. This Figure and Table (Figure IV and Table II) show the results of our *Rhizopus delemer* fungal culture that has positive results for the catalase enzyme synthesis. *Rhizopus delemer* fungi electro hydrolyse the Hydrogen peroxide into water and oxygen bubble in free form[43]. Normal state $H_2O_2$ is a hard compound. In biosystem, it irritates the respiratory tract and corrosion by the results in burning of skin and eyes[44]. In *Rhizopus delemer* endophytes, catalase enzyme act as an el- donor or acceptor in Reactive Oxygen species[45] and also a scavenger involved in many cellular defence mechanism[45]. Many industrial Applications are underlying in isolation of catalase enzymes from endophytes ex: Bioremediation of crude oil, Food industry, and medical cancer studies[46]. Research in *Saccharomyces cerevisiae*, Cat protein which is synthesis from catalase A and T genes, this report helps in analysis of catalase enzyme production in various upstream processing[47].

### *4.3 Phyto and Pharmacological activity of Bioactive compounds*

Table (Table III) shows the Qualitative Screening of Phytochemicals, here there are 12 bioactive compounds present. Which are Tannins, Saponins, Steroids, Terpenoids, Cardiac glycosides, Anthroquinones, Glycosides, Phenols, Alkaloids, Xanthoproteins, Emodin, Carbohydrates. These phytochemicals act as a defence mechanism against infectious disease[48]. Table and Figure (Table IV and Figure V) represent the GC-MS study of *Rhizopus delemer* fungal extract there are 20 bioactive compounds present. This Table (Table V) particularly explains the pharmacological activity of 9 bioactive compounds. In this 1,1,3-triethoxybutane compound act as anticancer[49] agent. The extract of *Monocardia vaginalis* this compound act as an effective molecule in liver and kidney necrosis. It was confirmed by histopathological studies. The 1,1,3-triethoxybutane as a Hydrophobic modifier in wastewater treatment[50]. The 3,7,11,15-tetramethyl-2-hexadecen-1-ol compound as antibacterial[51], antioxidant and anti-inflammatory in RAW 264.7macrophages cells. When it increased the value in the presence of nitric oxide while preparing the extract of leaf *Polygonum odortum*[52]. In Nano drug delivery systems, 1-hexadecane metal ligand compound maintain the self-assembly stability[53] in various reaction temperatures. Silane, environment friendly antimicrobial corrosion agent, coupling agent, adhesion promoters, and surface modifiers[54]. It is an Encapsulation compound[55], Because Silane made up of polymer of silicon (Si-C) carbon bonds. In nano-drug delivery system, silane releases Retinol encapsulation compound in treatment of eye. It protects the nano compound till the time of delivery. 1,2-benzene dicarboxylic acid compound used as a plasticizer[56,50] for various areas such as vinyl foams, which are often used as floor tiles, traffic cones, food conveyor belts and artificial leather. 2-methoxy-1-methyl propyl acetal compound is an antioxidant, anticancer[57] agent. Acetal-based nano-drug facilitate increased targeted drug delivery in low pH for ex in cancer treatment and bacterial infection. And also, this compound act as an

Antiphotoaging compound[58]. 3-ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris compound as an antifungal, antibacterial, antiviral agent[59]. (15)-n-labelled -diethylamine is an anti-cancer activity agent[60]. In cancer study, derivatives of (15n) labelled compound against to malignant SF268, MCF-7, NCI-H460 and skin mcf adenocarcinoma cell lines. Alpha-d-mannopyranoside, methyl 3,6-anhyd, derivatives are precursors of oligosaccharide synthesis[61].

## 5. CONCLUSION

Our findings and reports conclude that *Rhizopus delemer* UICC 26 strain is an endophytic fungus growing in *Abutilon indicum* plant leaves. The endophyte has plant growth promoting property by relations with *Rhizopus delemer* that was confirmed by Hydrogen cyanide test and Catalase enzyme test. It described plant growth were influenced by endophytic fungi *Rhizopus delemer* and also because of their presence in the host plant, Fungi produced the lethal active compound HCN and biocontrol agent of Catalase enzyme against pest, weeds, other herbivores and disease-causing microbes. It enhances the positive way in plant-endophyte symbiotic association. In Gas Chromatography-Mass Spectroscopy studies in *Rhizopus delemer* fungal extract producing secondary metabolites which could be biosimillar to the host plant and also extracted metabolites can be separated depending on the polarity and volatile nature. Bioactivity reveals extracted secondary metabolites were used for various medicinal and therapeutic purposes such as antimicrobial, anti-inflammatory, anticancer and antioxidant activity. In future beyond doubt, endophytic fungi *Rhizopus delemer* to create a breakthrough in Myco - pharma research field.

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## 7. AUTHORS CONTRIBUTION STATEMENT

Ms Deepalakshmi was involved in the collection of samples, techniques in the isolation of fungal cultures and preparation of data in GCMS, and the preparation of manuscript under the guidance and inputs from Dr Joseph Sebastin Raj and the final manuscript was prepared by incorporating inputs from both authors.

## 8. CONFLICT OF INTEREST

Conflict of interest declared none.

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