Anti-larvicidal antimicrobial and cytotoxic effect of endophytic fungus Alternaria macrospora isolated from Trichillia connaroides

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Abstract

The interaction of plants with microorganisms at various trophic levels is primarily for growth, development and deterrence and is intertwined in a complex network of inhabitants. Endophytes of fungal origin are widely recognized for supporting the production of host plant defence systems, new secondary metabolites, biological pest control agents, and beneficial colony-forming fungi. This study focuses on the assessment of the biological activity of the endophyte, *Alternaria macrospora* isolated from the leaves of *Trichillia connaroides*. This strain was genotyped by amplification of the internal transcribed spacer (ITS) gene region. Chemical profiling of the extract is performed by gas chromatography-mass spectrometry (GCMS), indicating the presence of functional groups by FTIR peaks. In addition, *A. macrospora* metabolites exhibit biological activity listed as antibacterial, antifungal, larval killing, and anticancer activities. It provides a catalogue of bioactivity that is highly relevant to drug and pharmacological settings.

Introduction

Endophytes (endo-internal, phytes-flora) are the microorganisms dwelling inside the other organisms without inflicting any soreness. Endophytic fungi asymptomatically live in the inner tissues of flowers, under the epidermis and colonizing the healthy dwelling tissues through quiescent infections (Wilson 2000). The endophytic fungal species are hosted in approximately 300,000 terrestrial flowers, each one web hosting one or greater endophytic forms. The endophytic strains were distinct from one of a kind varieties of flora: timber (yew, pine), fodders (clover, alfalfa, sorghum), vegetables, culmination, cereals and other crops (Lu et al. 2012). They play a critical function inside the enhancement of plant health and in-particular as bio-control agents in the suppression of plant pest groups like bugs and pathogens (Xiang et al. 2016).

Endophytic fungi are known to possess antimicrobials, anticancer compounds like Taxol a furnishing factors of significance in agriculture, pharmaceutical, medicine and nanotechnology (Tapwal et al. 2015). These endophytes are well known for the production of biologically active secondary metabolites namely, terpenoids, alkaloids, steroids, isocoumarins, quinones, phenylpropanoids, lactones, lignans and phenols (Santos et al. 2015). Endophytic fungal strains of *Pseudotaeniolina globosa* and *Penicillium purpurogenum* are said as larvicides (Seetharaman et al. 2017). *Trichoderma harzianum* is known to control tomato gray mould resulting from *Botrytis cinerea*, without fungicide resistance and environmentally benign (Pan et al. 2016). Of late, the endophytic fungal species *Penicillium citrinum* from *Digitaria bicornis, Diaporthe longicolla* from *Saraca asoca* were reported to possess antioxidant and antibacterial activities (Nishad et al. 2021); (Nischitha and Shivanna 2022). The filamentous fungi possess a few advantages: clean to handle; require easy vitamins, own excessive wall-binding capability and green intracellular metal uptake competencies (Dias et al. 2002).

Fungi synthesized secondary metabolites can also be an alternative to artificially formulated pesticides, as they are comparatively cheap and biodegradable (Lorenzen and Anke 1998). Microbes are a wealthy supplier of bioactive secondary metabolites and are an opportunity source of mosquito control agents like positive bacterial strains (*Bacillus thuringenesis var. israelensis (Bti) and B. sphaericus (Bs)*. These bacterial toxins are well-known for its excessive effectivity against mosquito larvae at a minimal dose and are inert and harmless to non-targeted organisms (Patil et al. 2011). Fungal strains belonging to the genera *Beauveria*, *Lagenidium*, *Coelomomyces*, *Culicinomyces* and *Metarhizium* are recognised to govern vector-borne diseases and a few pathogens (Kamareddine 2012).

The genus *Trichillia* (belonging to the family Meliaceae), comprising of about 419 plant species, is known to possess pharmacological and biological propensities. *Trichillia connaroides* (Wight and Arn), a previous nomenclature named as *Heynea trijuga* Roxb, has been used as a traditional remedy in South-East Asia, India and China. It is a small

evergreen tree widely distributed in South and East Asia including India, Indonesia and South China. In recent years, enormous research have been related to the insecticidal and pharmacological efficacy of *Trichillia sp.*, which possess biological activities like insect anti-feedant, insecticidal and insect growth regulators, along with anti-microbial, anti-malarial, anti-most cancers, antiviral and different pharmacologic uses (Roy and Saraf 2006). The main objectives of the present entities is to isolate and identify some endophytic fungi from the leaves of *Trichillia connaroides* through morphological and molecular techniques and to examine it's biologically potentialities.

Materials And Methods

Collection of plant material

Fresh leaves of *Trichillia connaroides* **(Figure 1**) were collected from the Botanical Survey of India (BSI) in Yercaud, Salem (District), Tamil Nadu, India. The collected plant samples were used to isolate endophytes.

Isolation of fungus from leaves

Fresh and healthy leaves of *T. connaroides* were collected and sterilized with various surfactants and washed thoroughly with sterile distilled water. After surface sterilization, the plant material was dried on sterile oil blotting paper and a manual method was used to prepare PDA medium containing the antibiotic chloramphenicol. The leaves were chopped to expose the inner surface of the plant, placed on a PDA plate and incubated at 27 ° C for 6 to 7 days. The incubation time for each fungal growth was recorded, the first visible growth day from the plating day was considered as the growth incubation period (Tolulope et al. 2015). Fresh PDA plates were prepared by isolating distinct fungal colonies from primary cultures and transferring them to fresh PDA plates. Inoculated plates were incubated in the dark at room temperature (27°C; 7 days) until further analysis.

Morphological identification of endophytes

Slide preparation

Morphological analysis (colony and spore morphology, colony texture, reverse pigmentation, hyphal structure, endogenous bacterial spores and spore chains) was performed by lactophenol cotton blue (LPCB) staining and observed the slides with a light microscope (Ranganathan and Mahalingam 2019).

DNA Isolation and PCR amplification

Fungal genomic DNA was isolated from fresh fungal endogenous mycelium by the cetyltrimethylammonium bromide (CTAB) method. The internal transcribed spacer (ITS) region was amplified using the universal primers ITS1 and ITS4. The whole reaction mixture was carried out in 40 µl consisting of template (4 µl), primer (10 µM; 2 µl each), Taq-PCR mix (20 µl) and double-distilled water (12 µl). Subsequently, the amplified region was further purified and sequenced using the QIA Rapid PCR Purification Kit (QIAGEN). Endophyte ITS sequences were compared to a database cataloged in NCBI using BLAST and phylogenetic relationships were performed by software CLUSTAL X (Venkateswarulu et al. 2018). The 5.8S rDNA nucleotide sequence was edited with Bioedit software and aligned with Clustal W software (Hall 1999). A total of 14 sequences were used in a phylogenetic analysis using the neighbor-joining method with 1,000 bootstrap replications based on the Kimura 2-parameter test of the MEGA 6.0 software (Tamura et al. 2011).

Extraction of Secondary Metabolites from Isolated Fungi

Mycelial mats from actively growing endophytic culture (a week old) was inoculated into 200 ml potato dextrose broth (PDB) medium and Incubated (28°C) at room temperature in the dark for 10-15 days. Once the maximal growth was reached and fungal mycelial mats were harvested by filtration. Mycelial extraction (10 g) was performed with 100 ml ethyl acetate under static conditions for 5 days. The extraction step was repeated 3 times and the mixture was filtered through a muslin cloth (Ragavendran et al. 2017).

Larvicidal and pupicidal activity of the endophyte *A. macrospora*

The collection and rearing of larvae and pupa

The first to forth instar larvae, pupa and eggs of *Aedes aegypti* at the developmental stage was collected from the ICMR Centre for Research in Medical Entomology in Madurai. The collected larvae, pupae and eggs were maintained in deionized water at 25 ± 2°C with a photoperiod of 10:14 L: D. The food contains dog biscuits, sucrose, and brewing yeast in a 4: 1: 1 ratio and is kept at room temperature for 24 hours before the start of the experiment (World Health (WHO 2005).

Larval and pupicidal bioassays

The experiments involving larval (1st, 2nd, 3rd and early 4th instars) and pupal stages were used for larvicidal and pupicidal bioassays according to WHOrganization (1981) protocol with slight modifications. Briefly, crude extracts (at various concentrations of 100, 300, 500, 700, 900 µg/µl) were prepared. Each test larva and pupa were placed in a 200 mL beaker filled with water. The test solution was dissolved in DMSO (1 ml) and tested against 1st to 4th instar larvae and pupae. The experiment was performed 3 times and tap water was used as a negative control. The number of dead larvae and pupae was counted in 24 and 48 hours, and the mortality rate was calculated using Abbott's formula (1925).

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Ovicidal bioassay

Ovicidal activity was determined by measuring inhibition of egg hatchability. *Ae. aegypti* oocytes collected from laboratory colonies were cultured in room conditions at 25-28°C. The newly laid eggs were observed with a stereomicroscope to evaluate the survival rate. Approximately 20-30 viable eggs were collected and exposed to various doses of ethyl acetate-soluble mycelial extract. The test was repeated 4 times and the negative controls (water cups) were kept separately. Results were recorded by observing egg hatchability 72 hours after treatment (Pineda-Cortel et al. 2019).

Dose response assay

The larvicidal, ovicidal and pupicidal bioassay were performed according to the World Health WHO (2005). A dose-dependent bioassay experiment was performed using selective endophytic mycelium extracts to analyze larval control, ovarian killing and pupicidal activity against *Aedes aegypti* larvae, eggs and pupae. Dose experiments (1-4 instars, pupae, and egg) were performed in 250 ml beakers using 6 different concentrations (100-1000 µg/ml) of metabolites. Negative controls was maintained in tap water and control mortality should not exceed 10% (Deepak et al. 2019).

Antimicrobial assay

Antibacterial activity

The bacterial strains, B. subtilis, S. aureus, E. coli and S. typhi were obtained from the Department of Microbiology at Mohan Kumaramangalam Medical College in Salem, Tamil Nadu, India. All isolates were maintained on nutrient agar medium (NA).

Agar well diffusion method

The antibacterial activity of the extracted bioactive metabolites was evaluated on human pathogens namely *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi* by agar well diffusion method. Pathogenic strains were spread on MHA (Mueller-Hinton agar) plates. Wells were made using sterile steel cork-borer and four different concentrations (25, 50, 75, and 100 µl) of fungal mycelium extracts were prepared and Incubated at 37 ° C for 24 hours. Negative (DMSO) and positive (Ciprofloxacin; 4 mg/ml) controls were taken and the inhibition zone (mm) was measured as mean ± SD (Sharma et al. 2016).

Antifungal activity

Sabouraud's dextrose agar (SDA) medium (Hi-media, Mumbai) was used to culture the pathogenic fungal strains, *A. oryzae*, *Trichoderma sp.*, *A. flavus*, *C. falcatum*. The freshly grown pathogenic cultures was spread evenly on the surface of the agar medium using a sterile cotton swab and a well (0.6 cm) was made. Ethyl acetate extracts of various concentrations (25, 50, 75, and 100 µl) were loaded into respective wells in each culture plates and incubated at 27°C for 48-72 hours. Fluconazole was used as a positive control. After incubation, the diameter of the inhibition zone was measured.

Anti-cancer activity

Maintaining cell line

HeLa (human cervical cancer) cell line was obtained from National Centre for Cell Sciences (NCCS)), Pune and grown in Eagle's Minimal Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). Cell cultures were maintained weekly at 5% CO_2, 37°C, 95% air and 100% relative humidity (RH) and medium was replenished twice every 7 days.

Cell line treatment

Monolayer cells were exfoliated with trypsin-EDTA to prepare a single cell suspension, and the viable cells were counted on a hemocytometer. Medium was diluted to a final density of 1 x 10^5 cells/ml using 5 % FBS. After seeding 100 µl cell suspension per well in 96-well plates with a plating density of 10,000 cells/well, for cell adhesion the plates were incubated at 37°C, 5% CO_2, 95% air and 100% RH. After 24 hours, the cells were treated with various concentrations of mycelial extract. First, they were dissolved in neat dimethyl sulfoxide (DMSO), followed by diluting the sample aliquots in serum-free medium to twice the desired maximum concentration. Additionally, the serial dilutions of up to 4-fold were performed for a total of 5 concentrations. Aliquots (100 µl) of these variable dilutions were added to the appropriate wells containing 100 µl medium and the final concentrations required were totalled. After adding the sample, the plates were incubated at 37°C, 5% CO_2, 95% air and 100% RH for 2 days. Sample-free medium was served as a control.

MTT assay

Chemical 3-[4, 5-Dimethylthiazole-2-yl] 2, 5-Diphenyltetrazolium bromide (MTT) cleaves the tetrazolium ring with the enzyme succinate dehydrogenase and converts it to insoluble purple formazan. The amount of formazan produced is

directly proportional to the number of viable cells, so the reaction was analysed. After 2 days of incubation, MTT (15 µl; 5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hours. The formed formazan crystals were solubilized in 100 µl DMSO and the absorbance was measured at 570 nm. Cell viability was calculated using the following formula:

Viability% = Test OD-Control OD/ 100

Gas chromatography and mass spectroscopy (GC-MS) analysis

The GC–MS analysis of fungal mycelial ethyl acetate extract was performed in electron ionization (EI) mode on GC–MS Perkin Elmer model 680 system (Switzerland). The initial temperature was set up at 70°C for 2 minutes; oven temperature at 280°C (at the rate of an increase of 5°C/min) was maintained for 9 minutes. The injection port temperature was maintained at 260°C with the flow rate of carrier gas (Helium) at 1 ml/min. The ionization voltage was kept at 70 eV and the samples were injected in split mode (10:1). The mass spectrum was obtained in the mass range of 40 to 1000 a.m.u (Ragavendran and Natarajan 2015). The compounds were identified based on the comparison of their retention time (RT) and mass spectrum using NIST library databases.

Fourier transformed infrared spectroscopy (FTIR) analysis

The purified and dried sample of ethyl acetate extract containing fungal secondary metabolites was analyzed using Shimadzu 8400S spectrophotometer, Shimadzu Corporation, Japan in the mid-IR region of 400-4000 cm^{-1}. The samples were prepared using spectroscopic grade potassium bromide (5:95). The functional groups present in the extract will be identified using standard IR reference chart (Rajalakshmi and Mahesh 2014).

Statistical analysis

Data were analysed using SPSS statistical software (20.0). The LC_{50} and LC_{90} values were calculated by Probit analysis with their lower and upper confidence levels (LCL and UCL). The level of significance (p-value) was fixed as < 0.05 (Buysse et al. 2004).

Results

Isolation and identification of endophytic fungi from *T. connaroides* leaves

The colonies of *Alternaria macrospora* were grayish green in color and white with septal hyaline and branched mycelium, solitary, dark brown, straight or slightly curved, oval conidia, with a long tapered beak. The size of the conidia is 50 to 87.5 x 7.5 to 17.5 µm, with 1 to 6 transverse septa and, 0 to 3 vertical septa, as shown in **Figure 1**.

Molecular identification of isolated culture

The isolated DNA sample was amplified under optimized conditions using universal primers and the rDNA fragment was shown in **Figure 2**. The obtained amplified products was located in the expected ITS region, exactly 552 bp. The resulting RNA sequence of the isolated coding 5.8S rDNA gene has been deposited with GenBank (NCBI) at accession number MN049541. Then, based on 5.8S rDNA sequence analysis, this strain showed 99% similarity to *Alternaria sp.,* of the previously reported sequence. The evolutionary history of isolates was biased using the neighbour-joining method **(Figure 3)**. The percentage of the replicate tree was calculated based on the related taxa clustered in the bootstrap test (1000 iterations) that appears next to the branch. For the phylogenetic tree analysis 14 different fungal ITS sequences were included.

Larvicidal activity using endophytic fungi *A. macrospora*

Endophytic fungi *A. macrospora* mycelial extract were tested for larvicidal activity against 1^{st} - 4^{th} instar larval stages of *Ae. aegypti*. The fungal extract exhibit superior toxicity against 1^{st} and 2^{nd} instar larvae of *Ae. aegypti* after 24 h exposure and reported 99% larval mortality. LC_{50} values of 1^{st} to 4^{th} instar larvae were found to be as 96.167, 131.370, 217.595, 143.864 µg/ml and followed by LC_{90} values figured at 395.16, 483.62, 882.545, 655.722 µg/ml (**Table 1)**. Also, the extract-treated 1^{st} and 2^{nd} instar larvae revealed a pronounced effect at the maximum concentration (700 µg/ml). More than 50% of larval deaths were reported within 8 h. Similarly, in 3^{rd} and 4^{th} instar larvae, the larval growth and development were arrested and affected. Among larval stages, dose-dependent growth inhibition was found to be in the order: 4^{th} instar > 3^{rd} instar > 2^{nd} instar > 1^{st} instar, respectively.

Table 1. Larvicidal and pupicidal effects of the *A. macrospora* mycelium extract against *Ae. aegypti*

Larva stages	Concentrations (µg/ml)	Mortality (%)	LC_{50} (µg/ml) (LCL-UCL)	LC_{90} (µg/ml) (LCL-UCL)	χ^2
1st instar	Control	0.0±0.0	96.167	395.16	4.976
	100	56.67±0.57	(46.786 –140.616)	(289.20-635.091)	
	300	76.67±0.57			
	500	90.0±0.00			
	700	100.0±0.00			
	900	100.0±0.00			
2nd instar	Control	0.0±0.0	131.370	483.692	7.973
	100	46.66±0.57	(80.182 –177.849)	(363.124- 753.16)	
	300	66.67±0.57			
	500	83.33±0.57			
	700	100.0±0.00			
	900	100.0±0.00			
3rd instar	Control	0.00±0.00	217.595	882.545	12.73
	100	36.66±0.57	(143.640 –288.134)	(622.50 – 1660.814	
	300	53.3±0.57			
	500	76.67±0.57			
	700	93.3±0.57			
	900	100.0±0.00			
4th instar	Control	0.00±0.00	143.864	655.722	10.24
	100	40.0±0.57	(82.494 – 199.248)	(471.998 –1154.365	
	300	56.67±0.57			
	500	73.3±0.57			
	700	96.67±0.57			
	900	100.0±0.00			
Pupa	Control	0.00±0.00	234.390	739.362	14.381
	100	33.3±0.57	(165.957 – 301.777)	(547.803-1222.998)	
	300	46.67±0.57			
	500	56.67±0.57			
	700	100.0±0.00			
	900	100.0±0.00			

LC 50 – Lethal concentration required to kill 50% of the population exposed; LC 90 – Lethal concentration required to kill 90% of the population exposed larvae, SD-standard deviation.

Pupicidal and ovicidal activity

In pupicidal activity, the percentage of mortality was calculated at different concentrations of mycelia extracts from the endophytic fungus *A. macrospora* against *Ae. aegypti* (**Table 1)**. The observed LC_{50} and LC_{90} values were found to be 234.390 and 739.362 µg/ml. The rate of mortality was calculated within the first 5 h of exposure which exhibited 60% mortality. A 100% mortality was observed in the pupae was reported after 48 hrs. The χ^2 value was significant at $p \leq 0.05$ level**.** Followed by ovicidal activity, the percentage egg hatchability of *Ae. aegypti* was treated with five different concentrations of *A. macrospora* mycelia extracts (**Table 2**). The toxicity induced by mycelial extracts was found to be concentration-dependent tested against *Ae. aegypti*. No hatchability (100% mortality) was recorded at 500 µg/ml compared to the control set (hatchability rate 96.8 to 100%). Also, LC_{50} and LC_{90} values were calculated as 36.080 and 204.422 µg/ml, respectively.

Table 2. Ovicidal activity of *A. macrospora* endophytic mycelium extract against *Ae. aegypti* eggs

Concentrations (µg/ml)	hatchability (%)	LC_{50} (µg/ml) (LCL-UCL)	LC_{90} (µg/ml) (LCL-UCL)	χ^2	Significance
Control	96.66±0.00	36.080	204.422	4.422	0.986
100	73.33±0.00	(2.390 – 77.706)	(110.541-355.100)		
300	56.66±0.00				
500	43.33±0.57				
700	23.33±0.57				
900	NH				

NH- No hatchability

Behavioural studies

The *A. macrospora* metabolites induced neurobehavioral toxicity on 4th instar larval stage of *Ae. aegypti*. The control larval set showed normal behaviour in contrast to the vigorous and restless movement of larvae noticed after 25 minutes exposure of to extract. With increased exposure time, the larvae exhibited altered movements, abnormal excitation and restlessness, forceful self-nibbling of mouthparts and anal papillae leading to the development of ring-shaped structures. Several larvae exhibited up-down wriggling movements, vibrating movements and paralytic symptoms.

Antibacterial activity of endophytic fungi *A. macrospora*

Antibacterial activity of mycelium extract was evaluated against four pathogenic bacterial strains, namely *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Bacillus subtilis.* The study reported inhibition of bacterial growth following a dose-dependent manner shown in **Table** 3. The maximum inhibitory zone was observed in *E. coli* (22 mm) **(Fig. 4**) followed by *S. aureus* (20 mm), *B. subtilis* (19 mm), and an average inhibition zone in *S. typhi.* No inhibition zone was observed in the negative control (DMSO).

Antifungal activity of endophytic fungi *A. macrospora*

The antifungal activities of *A. macrospora* mycelium extract against fungal pathogens were *Colletotrichum falcatum, Aspergillus flavus, Trichoderma sp,* and *Aspergillus oryzae.* The maximum inhibitory zone was observed in *Trichoderma sp* of (21 mm) and also it strongly inhibits *A. flavus* (20mm) were shown in **Figure 5**. The moderate inhibition zone was observed in *A. oryzae* while the poor inhibitory effect was observed against *C. falcatum* (12 mm) (**Table 4**). So this suggests that *Trichoderma sp* have better activity compared with other fungal strains. This result also indicated that the inhibitory effect is differed with fungal type, compared with the positive control.

Table 4. Antifungal activity of mycelium extract of endophytic fungi A. macrospora

S. No.	Microorganisms	Zone of inhibition (mm)				
		Flucanozole	Concentrations of ME (µg/ml)			
			25µl	50µl	75µl	100 µl
1.	*A. oryzae*	22±0.58	11±1.0	16±1.15	18±2.6	19±2.0
2.	*Trichoterma sp.*	28±1.0	12±1.53	15±1.0	18±1.53	21±1.0
3.	*A. flavus*	27±1.53	11±2.0	17±1.53	19±1.0	20±0.5
4.	*Colliotrichum falcatum*	Nil	Nil	Nil	9±1.0	12±1.5

Negative control (DMSO) – No activity; ME - mycelia extract

Cytotoxic activity

The cytotoxic activity of fungal endophyte was tested by MTT assay on HeLa (human cervical cancer) cell line. Cells were treated with variable concentrations of fungal extracts (50- 300 µg/ml) and changes in cell shape were observed under an inverted microscope. The normal cells were usually short and round but the treated cells exhibited noticeable morphological changes like cell shrinkage, a decrease of cell volume and loss of adhesion (**Figures 6 & 7**). The IC_{50} value of *A. macrospora* mycelial extract was found to be 118.49 µg/ml.

Gas chromatography and mass spectroscopy analysis

GC-MS analysis of *A. macrospora* fungal extract produced some bioactive compounds. The identification of compounds was done based on NIST databases by the virtue of their comparisons to actual mass spectral data (**Fig. 8 & Table 5**). Here, totally seven compounds were identified: Phenol, 3,5-bis(1,1-dimethylethyl) (4.572%), 3-isopropoxy-1,1,1,7,7,7- hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane (4.453%), Dihydroartemisinin, 10-o-(t-butyloxy (8.33%), Cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl) (15.284%), Oleic acid (22.093%), Eicosanoic acid (9.407%), Ether, 6-bromo-1-ethyloctyl methyl. The chemical structure of these compounds were depicted in **S. Fig. 1.** The compound oleic acid has been reported to possess good larvicidal and anticancer activity.

Table 5. Identification of secondary metabolites from ethyl acetate extract of *A. macrospora* by GC-MS analysis

S.No	Name of the compound	R_T	Area%	Mol. form	Mol. wt	Biological activity	References
1.	Phenol, 3,5-bis(1,1-dimethylethyl)	29.48	4.572	$C_{14}H_{22}O$	206	Antimicrobial activity	Fabio Granados *et al*., (2016)
2.	3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	25.54	4.453	$C_{18}H_{52}O_7Si_7$	76	Antimicrobial activity	Gupta *et al*., (2017)
3.	Dihydroartemisinin, 10-o-(t-butyloxy)-	26.93	1.794	$C_{19}H_{32}O_6$	56	Anticancer activity	Chan *et al*., (2013)
4.	Cyclohexanol, 4-ethenyl-4-methyl-3- (1-methylethenyl)-, [1R-(1α,3α,4β)]-	23.23	15.284	$C_{12}H_{20}O$	180	-	-
5.	Oleic acid	22.37	22.093	$C_{18}H_{34}O_2$	282	Larvicidal & Antioxidant activities	Wei *et al.* (2016) and Fontana *et al*. (2013)
6.	Eicosanoic acid	21.17	9.047	$C_{20}H_{40}O_2$	312	Larvicidal and Antioxidant activities	Silva *et al*., (2016)
7.	Ether, 6-bromo-1-ethyloctyl methyl	24.78	4.182	$C_{11}H_{23}OBr$	250	-	-

Fourier transform infra-red spectroscopy analysis

The FT-IR spectrum (4000-400 cm^{-1}) of ethyl acetate extract of *A. macrospora* confirmed the presence of various functional groups of different organic compounds (**Fig. 9 & Table 6**). The sharp and strong spectrums indicated bands at 3317.54, 2924.09, 2854.65 cm^{-1} were assigned to C-H stretching vibrations of alkynes and alkyls groups. The broad peak at 1710.86 cm^{-1}correspond to the C=O stretching of carboxylic acid present in the sample. The medium band at 1656.85 cm^{-1} can be described as C=C stretching vibration indicating the presence of alkenes groups. The strong band could be assigned at 1305.81cm^{-1}, 1452.40 cm^{-1} corresponded to C-H bend and C-F stretch provide alkyls and halides groups. A peak at 1232.51 cm-1 denotes the alkyl halides (C-F stretch). The absorption bands have exhibited the presence of the ethers group at 1259.52 cm^{-1} (=C-O-C stretch). The peak at 1163.08 exhibited the presence of esters (O=C-O-C stretch). The medium peak at 1099.93 showed the presence of alcohol (C-O stretch) groups. The strong peaks of 844.82, 800.46 cm^{-1} indicate the alkyl halides (C-Cl stretch). A peak at 698.23 cm^{-1} revealed the alkenes (=C–H bend) respectively.

Table 6. Identification of functional groups from ethyl acetate extract of *A. macrospora* by FTIR

S. No	Wavenumbers (cm^{1})	Vibration	Visible intensity	Functional groups
1	3317.54	C-H stretching	Sharp	Alkynes
2	2924.09	C-H stretching	Strong	Alkenes/Alkyls
3	2854.65	C-H stretching	Strong	Alkenes/Alkyls
4	1710.86	C=O stretching	Broad	Carboxylic acids
5	1656.85	C=C Stretching	Medium	Alkenes
6	1452.40	C-H Bend	Strong	Alkanes/Alkyls
7	1305.81	C-F Stretch	Strong	Alkyl/Halides
8	1259.52	=C-O-C Sym and Asym: stretch	Medium	Ethers
9	1232.51	C-F Stretch	Strong	Alkyls/Halides
10	1163.08	O=C-O-C Stretch	Strong	Esters
11	1099.93	C-O Stretch	Medium	Alcohols
12	844.82	C-Cl Stretch	Strong	Alkyls Halides
13	800.46	C-Cl Stretch	Strong	Alkyls Halides
14	698.23	=C-HBend	Broad	Alkynes

Discussion

Endophytes are known to be the producers of various secondary metabolites (Vijayan and Balaraman 1991). In nature, their abundance far exceeds the numbers of other microbes, as they are reported by a variety of sources, from hostile marine environments to the harsh terrain of deserts and ice caps. Traditionally, most fungal species are identified based on their morphological characteristics (Rekha et al. 2015). Isolated fungi were identified by routine morphological and cultural features such as textures and colonies with reduced sporulation, colour, odour, and other sensory features (Barseghyan and Wasser 2010). In addition, methods for identifying endophytes by polymerase chain reaction (PCR) and DNA sequencing are well discussed. Identification of endophytic fungi at the molecular level is usually done by amplification of the internal transcription spacer (ITS) region (Innis et al. 2012).

In this study, the endophytic *Alternaria macrospora* was isolated from the leaves of *Trichillia connaroides* and identified based on fungal morphology, microscopic features and molecular analysis. In previous reports, the endophytes *Aspergillus*, *Cladosporium*, *Fusarium*, *Phomopsis*, *Colletotrichum*, *Alternaria*, *Diaporthe*, and *Trichoderma* were identified based on colony morphology and molecular analysis (Mbilu et al. 2018). Similarly, endophytes *Gliocladium solani* and *Penicillium melinii* from *Artemisia scoparia* with white colonies have been reported (Nisa et al. 2018). In our study, the endophyte *A. macrospora* showed whitish colonies. In the context of this study, the colony morphology of *Alternaria officinalis*, previously isolated by the study group, shown to produces wool-like pale gray septal hyphae with linear and curved conidia arranged in linear and bifurcated chains (Ranganathan and Mahalingam 2019). In this study, *A. macrospora* colonies were grayish green in color and were septal, vitreous, straight and branched mycelia. In addition, molecular phylogenetic analysis of the 5.8S RNA ITS (500 bp) sequence confirmed that the isolated strain was *Alternaria macrospora*. Using a combined approach of morphological and molecular

confirmation, similar reports on the phylogenetic identities and relationships of endophytic fungi isolated from *Artemisia indica*, *A. capillaris*, and *A. lactiflora* plant species have been previously documented (Huang et al. 2009).

Different groups of endophytic fungi are known to be pioneering producers of bioactive molecules with different configurations and structural arrangements. It was a known fact that various organic compounds derived from endophytes have biological activities such as antibacterial, antifungal, antitumor, antioxidant, anti-inflammatory and larval control (Selvi 2014). Several endophytes from various medicinal plants have contributed to the fight against emerging infectious diseases (Sharma et al. 2016); (Kim and Ahn 2017). In this study, the ethyl acetate extract of *A. macrospora* shows high mortality of *Ae. aegypti* larvae and is sensitive to compounds contained in mushroom extract. This may supports the research aimed at developing new mosquito repellents based on bioactive compounds from fungi on behalf of chemical larvicides (Abutaha et al. 2015). It was found that most endophytes have excellent larval control activity. Thus, *C. ptroselinum* for *Culex pipiens* larvae showed a clear LC50 value of 152.94 µg / ml (Khater and Shalaby 2008). A novel isoquinoline from *F. moniliforme* for *Ae. aegypti* and *A. stephensi* showed LC_{50} of 237.0-276.4 ppm (Pradeep et al. 2015). In this study, LC_{50} and LC_{90} values were found to be 96.167 to 143.864 µg / mL and 395.16 to 655.77 µg / mL, respectively, for the 1st to 4th instar larvae of *Ae. aegypti*. Similar studies have been reported on the mosquito and larval-killing properties of the fungus *A. terreus* against *An. stephensi* and *Ae. aegypti* (Ragavendran and Natarajan 2015). Vyas et al. (2007) have found 100% mortality when the metabolites of the fungus *Lagenidium giganteum* treated with 1st instar larvae of *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi*. In our study, an extract of 700 µg / ml *A. macrospora* showed 100% mortality in 1st instar larvae. Dharumadurai et al. (2010) have reported 23 isolates of actinomycetes, in which *Streptomyces sp*. showed remarkable activity of against *Anopheles*. Wherein, the present study the LC_{50} and LC_{90} values for mycelial extracts from *A. macrospora* ranged from 234.390 to 739.362 µg/ml with the Impact on pupal development of *Ae. aegypti*. Similar report by Murugan et al. (2011) stated the insecticidal and pupicidal effects of *M. anisopliae* against the malaria mosquito *An. stephensi*. Ragavendran et al. (2017) have analyzed the insecticidal and pupicidal effects of entomopathogenic fungus *B. bassiana* against *Ae aegypti*, *Cx. quinquefaciatus* and *An. stephensi*. The macrospora extract also showed promising ovicidal activity due to the bioactive metabolites present in the extract. Al-Mekhlafi (2018) has reported on the ovicidal activity and histopathological changes induced by the *Carum copticum* extract against *Cx. pipiens*. In our study, 100% ovicidal activity was observed in *Ae. aegypti* and LC_{50} was 36.08 µg/mL. The rate of hatching was found to be inversely proportional to the concentration of the extract. Reegan et al. (2013) has reported LC_{50} value of 62.5 ppm of *Clionacelata* extract for eggs of *Cx. quinquefasciatus* and *Ae. aegypti*. MARTÍNEZ et al. (2007) have screened the Ethanol extracts of five sponges, *Ircinia campana*, *Amphimedon compressa, Agelas sventres*, Topsentia ophiraphidites, and Svenzeazeai, were screened against larvae and eggs of *Cx. quinquefasciatus* and *Ae. aegypti*.

The antibacterial activity of mycelial extracts has been reported in many studies (Sharma et al. 2016). Santos et al. (2015) have reported the endophytes from the leaves of *I. suffruticosa* against bacterial pathogens such as *Bacillus subtilis* and *Escherichia coli*. In our study, it was found that *A. macrospora* mycelium extract strongly inhibits *E. coli* (22mm), *B. subtilis* (19 mm), *S. aureus* (20 mm) and *S. typhi* (16 mm). Makut and Owolewa (2011) reported the inhibitory activity of the endophytes *A. flavus* and *A. niger* against *S. aureus* and *E. coli*. Petit et al. (2009) have studied the activities of *Penicillium sp.* against *Streptococcus pyogenes*, *S. typhimurium* and *B. cereus*. In this line, the endophytic fungi *D. longicolla* resulted the antibacterial activity on challenging MRSA (22 mm) with MIC of 40 µg/mL (Nishad et al. 2021). Endophytes are known to produce antifungal compounds that can be extracted with ethyl acetate (Donald et al. 2005). Ethyl acetate extract exhibits strong antifungal activity. This may be due to an antifungal compound that was secreted into the culture medium and previously retained intracellularly. Herein, we have found that the active antifungal compounds have been produced by *A. macrospora* against the fungal pathogens *C. falcatum*, *A. flavus*, *Trichoderma sp*. and *A. oryzae*. Premjanu et al. (2016) have noticed that the largest inhibitory zone

against *Malassezia pachydermis* from *A. niger*. In a crude extract of *Alternaria sp.*, the compound oleic acid was detected with antifungal property against phytopathogenic species of *Moniliophthora perniciosa* and *P. ultimum* (Walters et al. 2004). In agreement with the earlier findings, the oleic acid compound present in the mycelial extract of *A. macrospora* exhibited excellent antifungal activity against *Trichoderma sp* and *C. falcatum.* Similarly, *Streptomyces diastaticus* isolated from *Portunus sanguinolentus* produced significant antibiofilm activity on *Candida albicans* by stating their active principles of Phenol, 3,5-bis(1,1-dimethylethyl) and Eicosanoic acid (Siddharthan et al. 2020). Thus, the possible interventions of these antifungal and antibacterial properties could be attributed by the secondary metabolites from *A. macrospora*

In this study, the anticancer activity of mycelium extract against HeLa cell lines was tested by MTT assay. The fungal extract showed 60% cytotoxicity at a concentration of 150 µg / ml and was significantly active against cancer cells (IC50 value 118.49 µg / ml). Kumari et al. (2018) have reported the cytotoxic activity of the endophyte *Talaromyces purpureogenus* against HeLa and MCF7 cell lines. Similarly, Artika et al. (2017) have reported the anticancer activity of the endophyte *Phomopsis sp*. against an MCF7 cell line with an IC50 of 19.2 µg/mL. Ragavendran and Natarajan (2015) studied the ethyl acetate extract of *A. terrus* and its components, where better insecticidal and pupicidal effects of the compound (phenol, 3,5bis1,1 dimethylethyl) on *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti.* In support, the presently revealed compound Phenol, 3,5-bis 1,1-dimethylethyl (4.57%) from the *A. macrospora* might exerted the antifungal activity. Likely, Sharma et al. (2016) have evaluated the bioactive secondary metabolites isolated from the endophyte *Pestalotiopsis neglecta* contained oleic acid as similar to the GCMS of *A. macrospora* extract i.e., oleic acid (22.093%) with a higher peak than other compounds. The GCMS analysis of the endophyte *Curvularia aeria* contained Eicosene (7.93%) which is quite similar to the peak area (9.047%) of the presently revealed icosanoic acid from *A. macrospora* (Sahani and Thakur (2019). FTIR is commonly used to identify the functional groups of chemical constituents and helps to elucidate compounds (Starlin et al. 2012). Our FTIR results showed the presence of phenolic compounds, alkenes, and amines based on wavenumber, peak assignment, and vibration mode. Similarly, CH stretches of alkene groups in ethyl acetate extracts of the endophytes *Chaetomium globosum*, *Penicillium janthinellum*, and *Cladosporium tenuissimum* were reported from an earlier study at 2855.08 cm^{-1} (Kanjana et al. (2019). The present mycelium extract of *A. macrospora* extract also confirmed the corresponding alkene group (CH stretch) at 2854.65 cm^{-1}. Furthermore, the main peaks of 3317.54 and 2924.09 cm^{-1} were assigned to the functional groups of alkyl (= CH stretch), which resembles intense peaks (3300.20 and 2924.09 cm^{-1}) representing aromatic and alkene-based CH stretches from the crude extract of *C. gloeosporioides* (Rabha et al. (2015)

Conclusion

Overall, this present study shed light on the endophytic fungus *Alternaria macrospora* isolated from the medicinal plant *Trichillia connaroides* based on both conventional morphological (colony morphology and conidia structure) and molecular (ITS region amplification) approaches. The ethyl acetate extract of *A. macrospora* shows good larval-killing toxicity to 1st to 4th instar larvae of *Ae. aegypti*, with LC50 values 96.17, 131.370, 217.595, 143.864 and 234.390 µg /ml; LC90 values : 137.370, 483.692, 882.545, 655.722, 739.362 µg /ml, after 24 h treatment. Followed by, the total mortality of pupae at the maximum concentration of mycelium extract (800 µg/ml) was recorded with the lethal concentrations (LC_{50} 131.370 and LC_{90} 483.69 µg/ml). The ovicidal activity of *A. macrospora* extract was also quantified with an LC_{50} value of 36.080 µg/ml. In addition, the endogenous mycelium extract showed broad-spectrum antibacterial activity against selected human pathogens. In addition, the fungal extract resulted the IC50 value of 118 µg/ml on HeLa cells using MTT assay, indicating that the extract is cytotoxic in nature. GCMS analysis showed the presence of potential secondary metabolites namely Cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl) (15.284),

Eicosanoic acid (9.047), Ether, 6-bromo-1 ethyloctyl methyl (4.182) and oleic acid (22.093%). Further, the functional groups were identified from the *A. macrospora* using FTIR spectrum.

Declarations

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Author contributions

Ragavendran Chinnasamy -Writing-original draft**, Balasubramani Govindasamy–**Formal analysis**, Tijo Cherian-**Investigation**, Natarajan Devaraja**n**–** Grammatical correction**,** Software, Resources.

Data Availability

The data presented in this study are available on request from the corresponding author.

Conflict of interest: The authors declare no competing interests.

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Figures

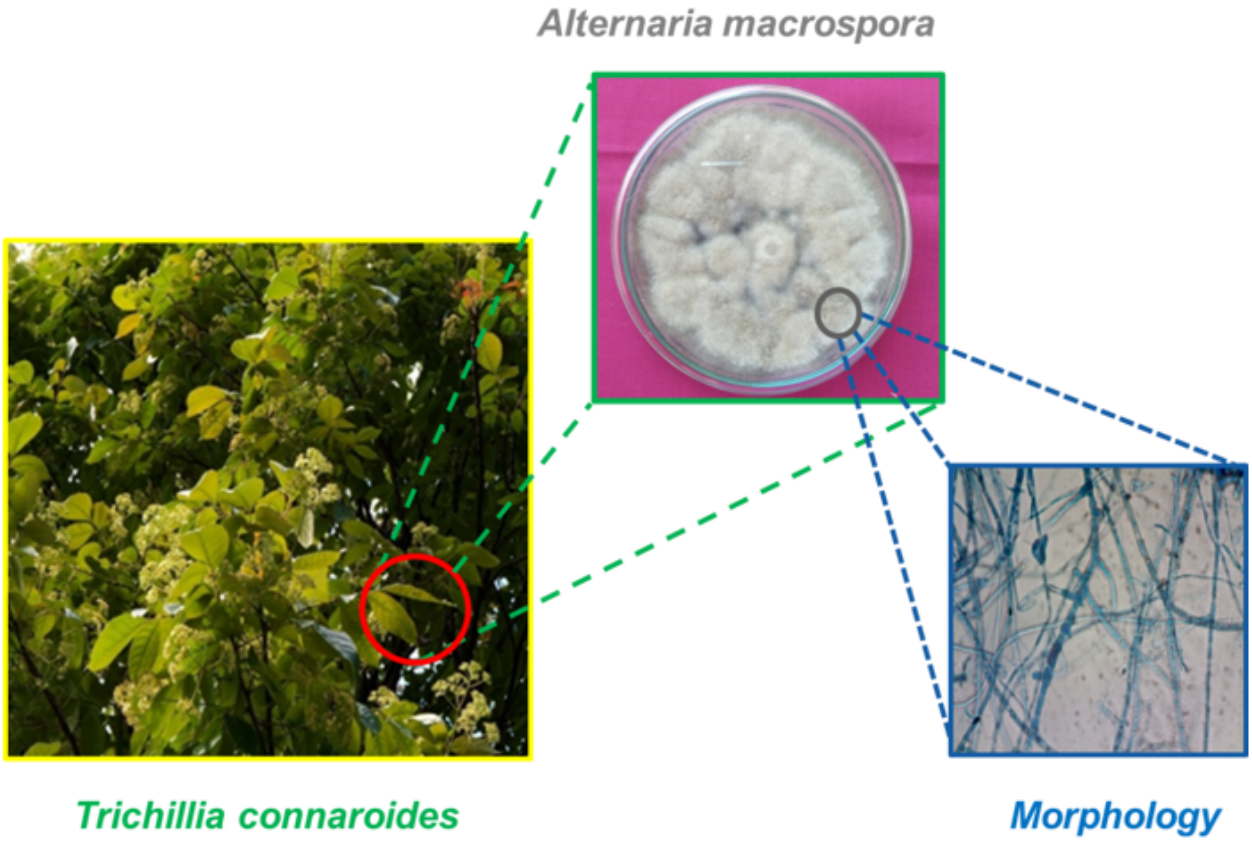

Figure 1

Photography of the plant *Trichillia connaroides,* the fungal species *Alternaria macrospora* isolated and its microscopic view showing morphoogical characteristics.

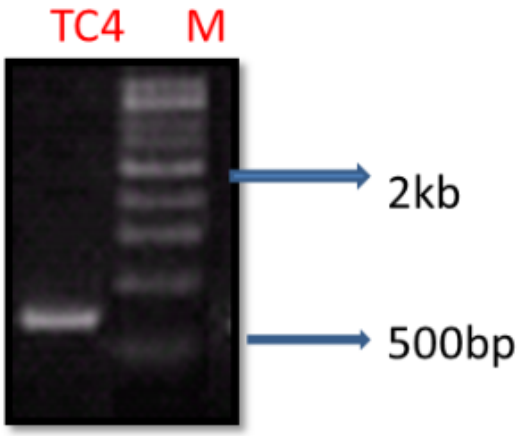

Figure 2

PCR amplification of isolated genomic DNA at ITS regions (ITS 1 and ITS 4)

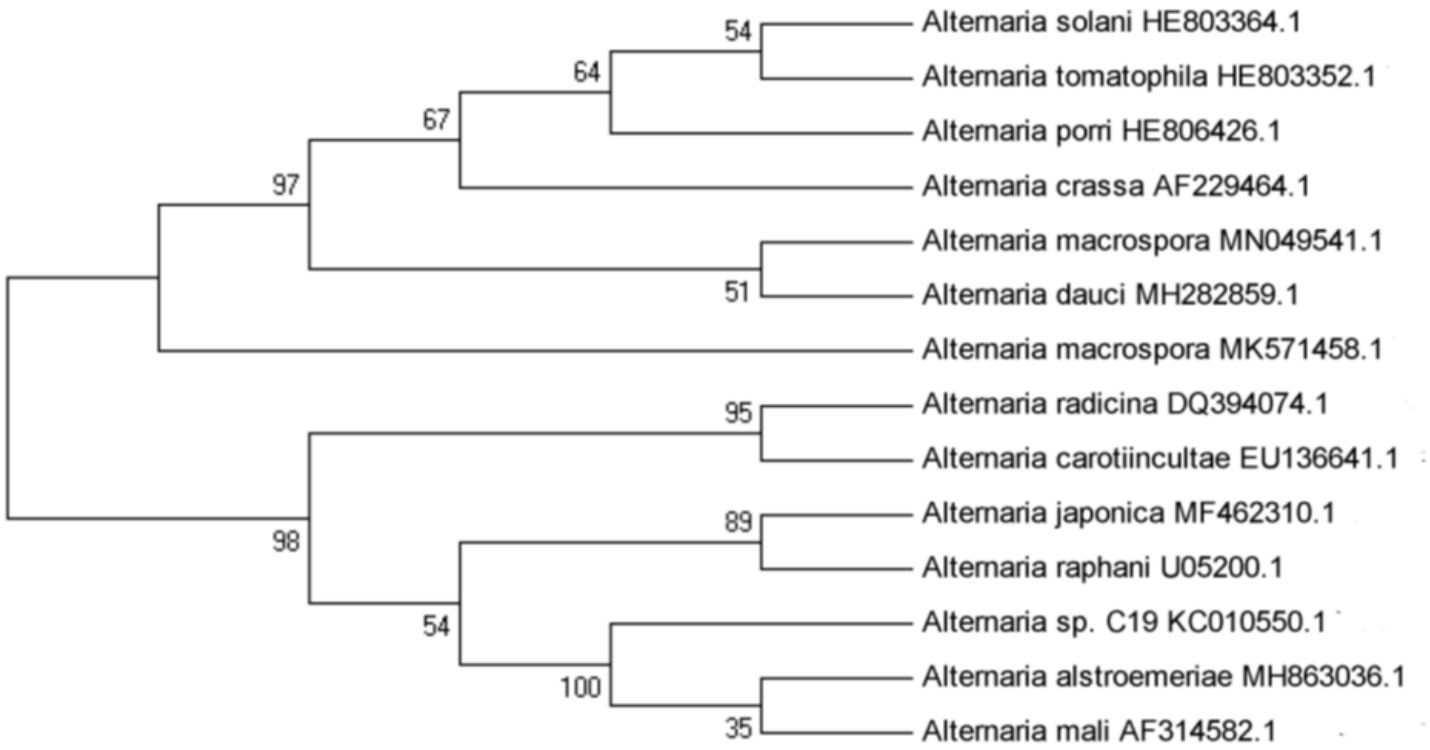

Figure 3

Phylogenetic tree analysis of *A. macrospora*

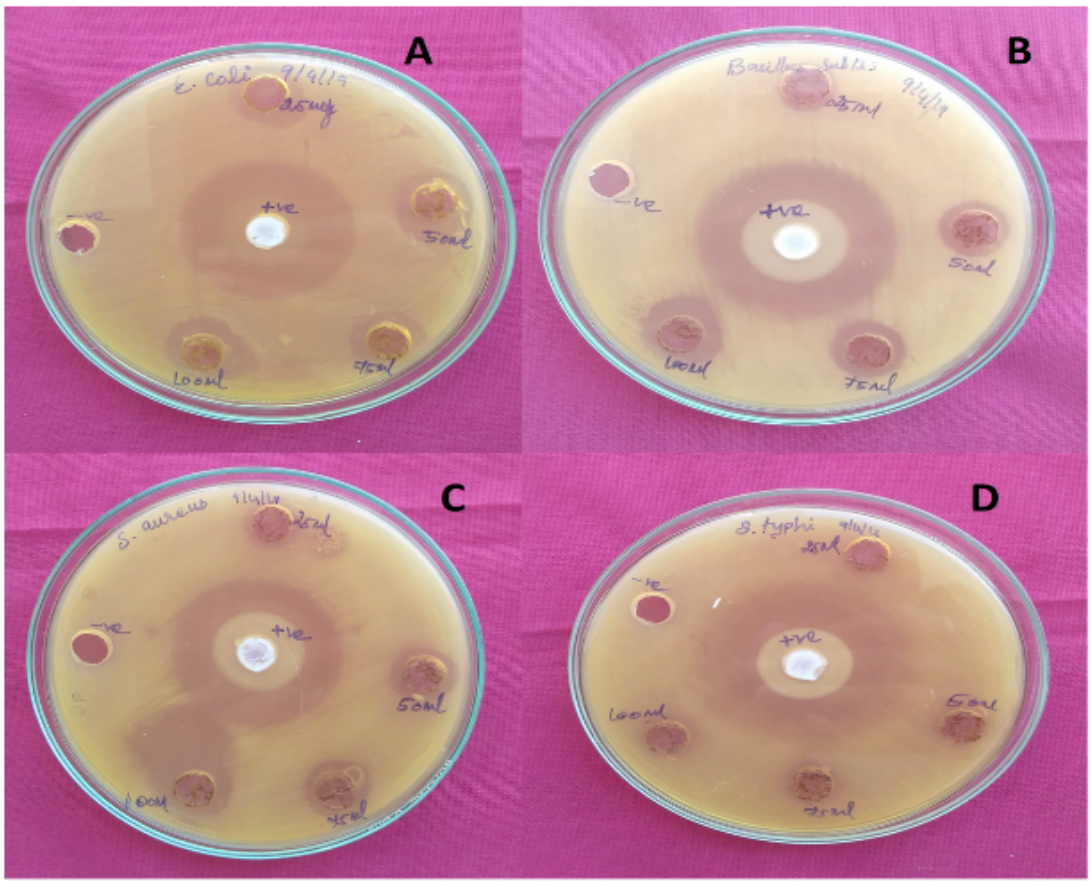

Figure 4

Antibacterial activity of the endophytic *A. macrospora* extract

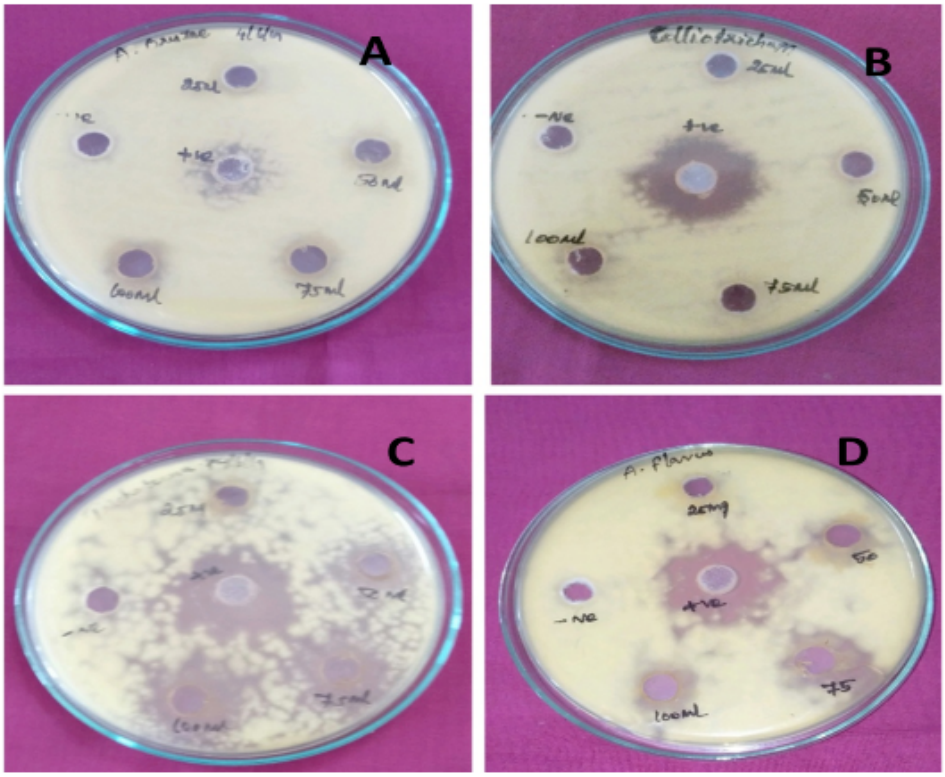

Figure 5

Antifungal activities using endophytic fungi *A. macrospora* mycelial extract **A**) *A. oryzae,* **B**) *C. falcatum,* **C**) *Trichoterma sp.* and **D)** *A. flavus*

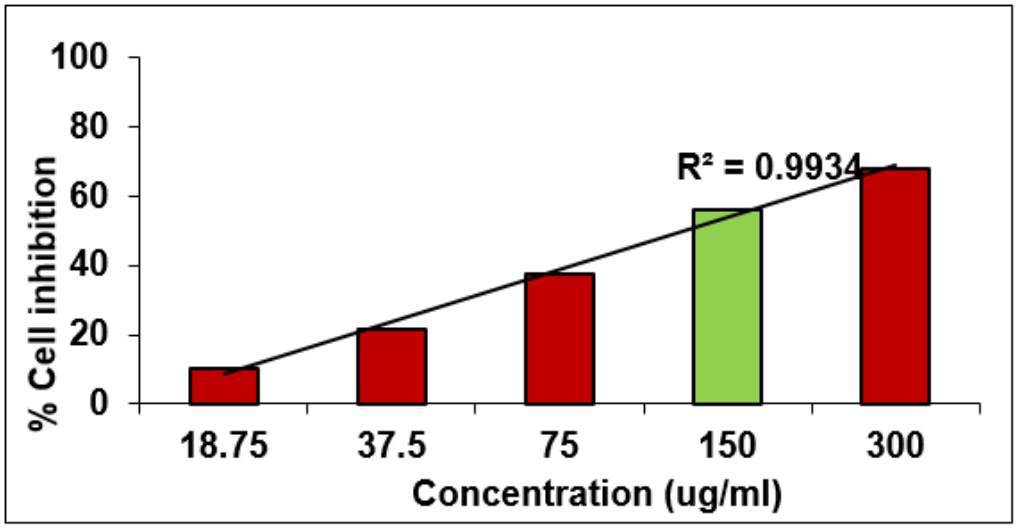

Figure 6

IC_{50} value of HeLa cell line treated with different concentration of *A. macrospora*

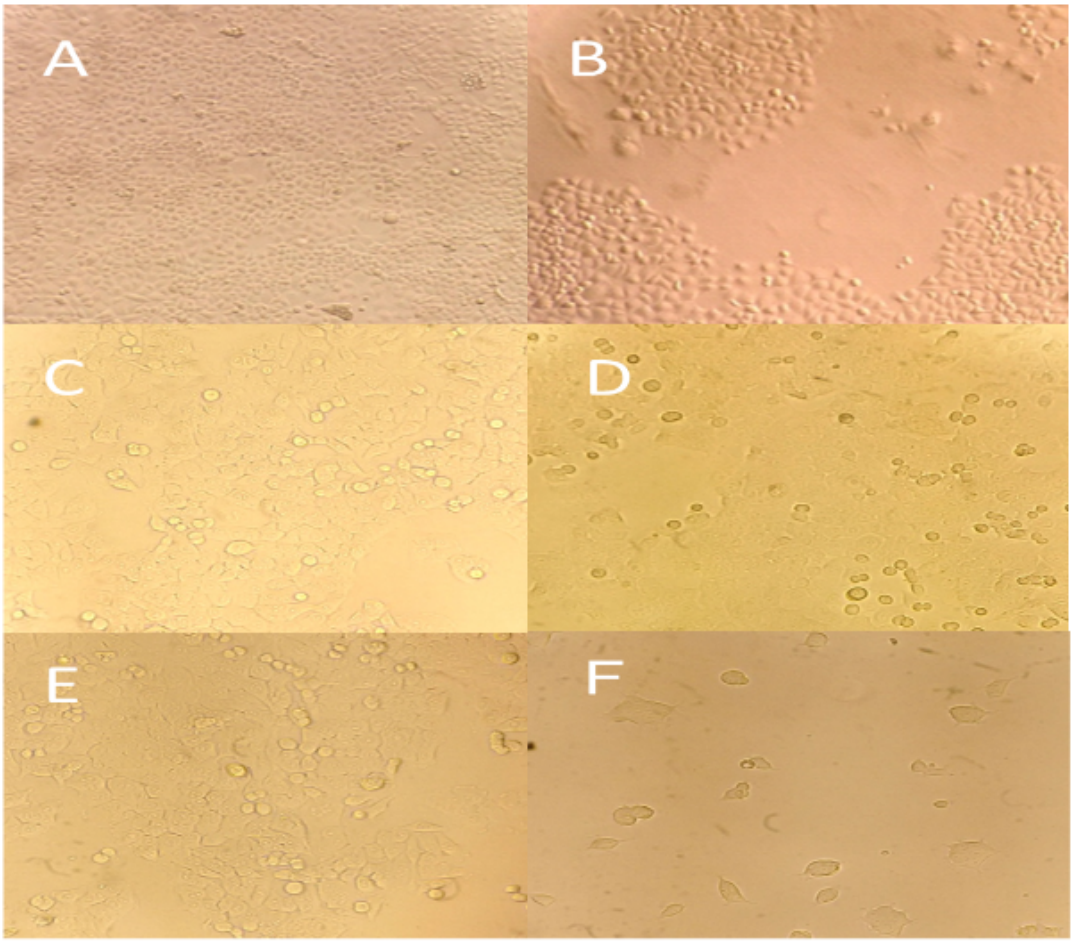

Figure 7

Cytotoxicity effect of ethyl acetate extract of A. macrospora against HeLa cell line (A) HeLa control cell line; (B) Mycelial extracts 18.75µg/ml; (C) 37.57µg/ml; (D) 75µg/ml; (E) 150 µg/ml and (F) 300µg/ml.

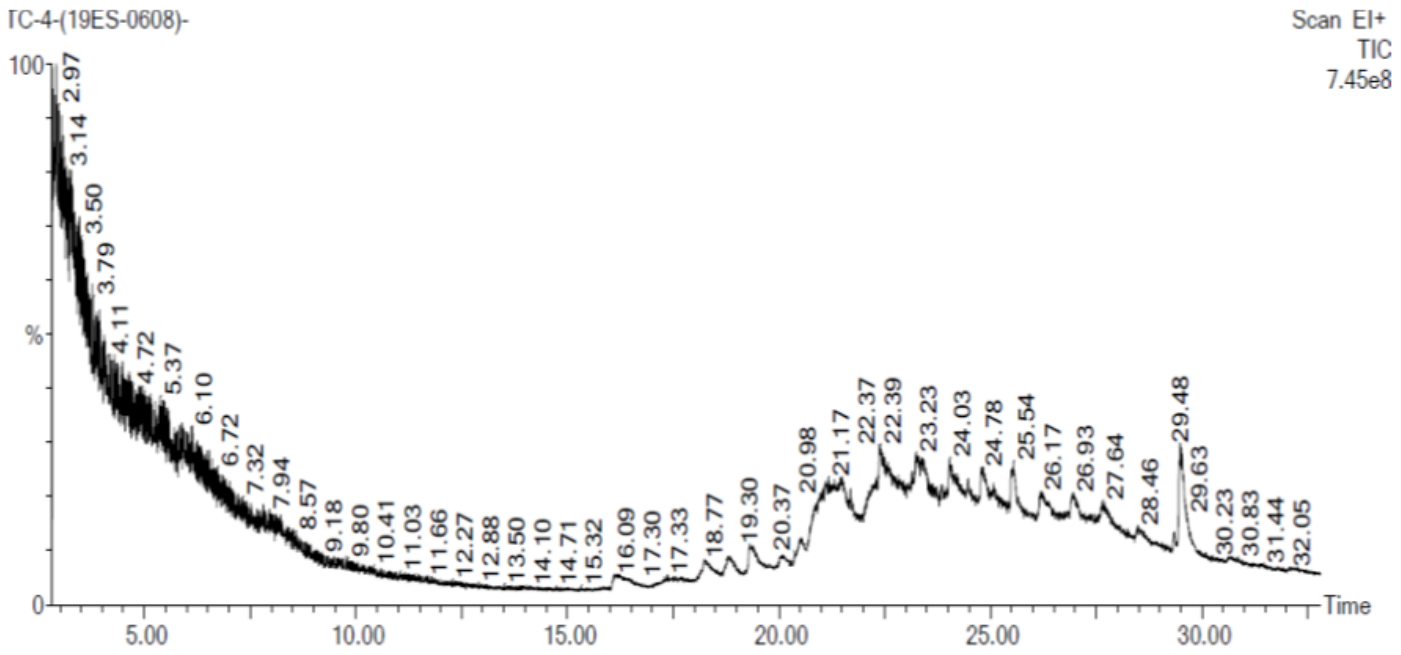

Figure 8

GC-MS analysis of ethyl acetate extract of *A. macrospora*

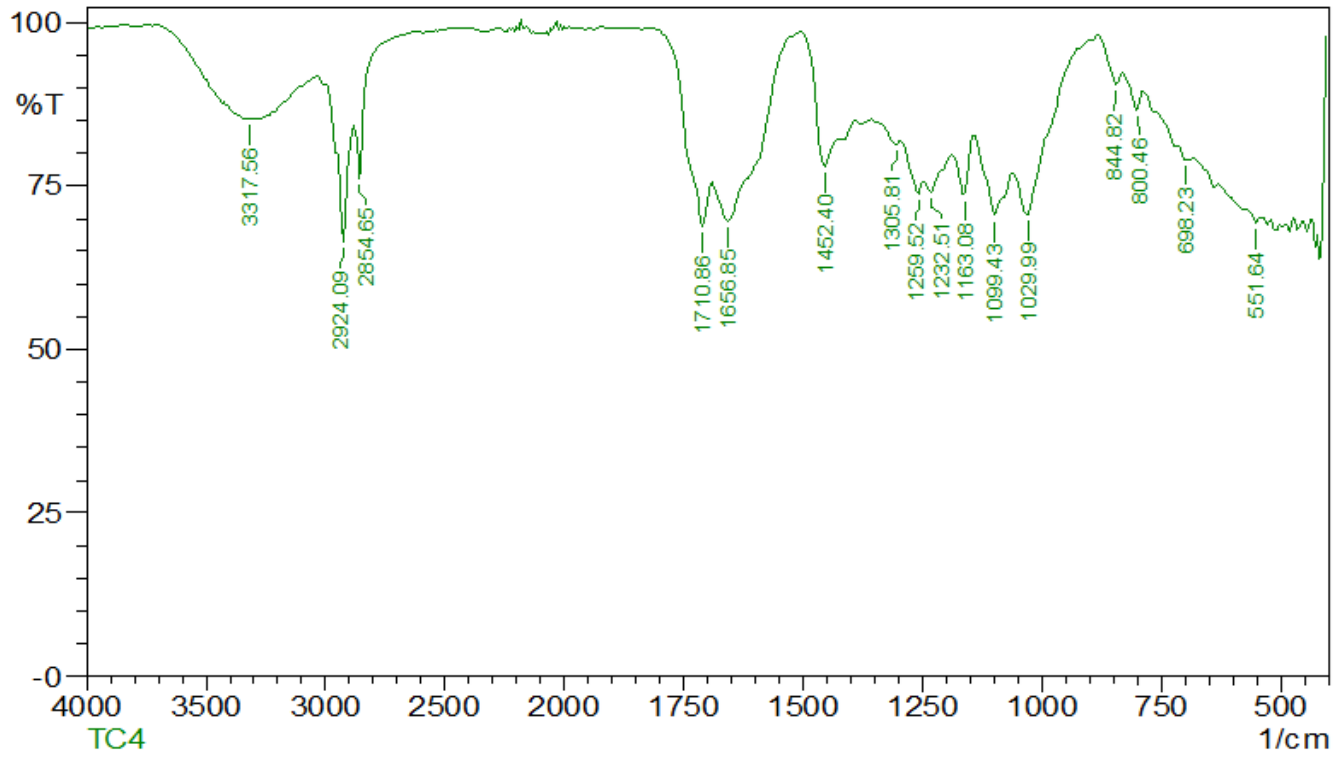

Figure 9

FTIR analysis of ethyl acetate extract of *A. macrospora*

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- s1.png