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Isolation, identification, of endophytic fungi from *Morinda Correia*, their invitro antimicrobial activity towards human pathogens and GC-MS analysis of bioactive compound

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Abstract
The present study aims to isolate endophytic fungi present in *Morinda correia* and screen for their antimicrobial activity. *Colletotrichum gloeosporioides* and *Alternaria tenuissima* were isolated from leaves of *M. correia* and identified through morphological and molecular methods. Both the organism were grown in Czapek'sdox broth medium and extracted with ethyl acetate. The ethyl acetate extract was tested for antimicrobial activity by disc diffusion method. Maximum inhibitory activity was identified towards *Staphylococcus aureus* by extract of *C. gloeosporioides.* Further investigation was carried out to identify the bioactive components produced by *C. gloeosporioides* using GC-MS. This study demonstrates the potential of isolated endophytic fungi from *M. correia* for the control of human pathogens.

Keywords: endophytic fungi; antimicrobial activity; *Morinda correia*; human pathogen

1. Introduction
The failure of currently used antibiotics required the search for new and effective antimicrobial agents. Hence, there is a growing attention in seeking new bioactive compounds with low toxicity, high efficacy, and low environmental impacts. In recent years, Endophytes have acquired the attention of the researchers because of their ability to produce novel bioactive metabolites with antibiotic, anticancer, antioxidant, and anti-inflammatory activity (Aly *et al*., 2010; Strobel *et al*., 2003; Tan and Zou 2001; Stinson *et al*., 2003; Kim *et al*., 2004; Ganga Devi and Muthumary 2007) [1, 2,3, 4, 5, 6]. Microorganisms that naturally reside inside the plants are known as endophyte, which is less susceptible to natural and external ecological imbalances without harming the host plant. Such microorganisms can be served as biological control agents that are appropriate in the environment (Mmbaga *et al*., 2018) [7]. Endophytes are biochemical synthesizers (Owen and Hundley, 2004) [8] synthesize various bioactive compounds (Patil *et al*., 2012; Schulz *et al*., 2002) [9, 10]. These bioactive compounds are used as pharmaceutical and agrochemical products (Petrini *et al*., 1991; Bacon *et al*., 2000) [11, 12]. An endophytic fungus *Microdochium bolleyi*, showed antifungal activity against a phytopathogen *Microbotryum violaceum* (Zhang *et al*., 2008) [13]. *Penicillium oxalicum*, an endophytic fungus isolated from *Gymnema sylvestre* which produces gymnemagenin used in the pharmaceutical industry as an antidiabetic agent (Parthasarathy and Sathiyabama, 2014) [14]. In this study, fungal endophytes were isolated from leaves of *Morinda correia* and they were identified by morphological and molecular method and evaluated for antimicrobial activity under In vitro condition.

2. Materials and Method
2.1 Sample collection and fungal isolation
The plant material was collected from Sirumalai in Dindigul district. The plant leaves were washed in running tap water for 10 min and subsequently washed with sterile distilled water and processed as follows: plant leaves were surface-sterilized by immersing in 0.5% (v/v) sodium hypochlorite for 3 minutes, then soaked in 70% (v/v) ethanol for 1 min, finally washed in sterile distilled water three times and surface dried with sterile filter paper as described earlier (Parthasarathy and Sathiyabama, 2015) [15]. After surface sterilization, the leaves were cut into 1 cm long segments and placed in an upside-down position in PDA plates amended with chloramphenicol (150mg/l). The plates were incubated at 25°C for 10-15 days. The fungus emerged from the cut end of leaf segments were transferred to new PDA plates without antibiotics. Stock cultures were stored at 4°C for further study.

2.2 Identification of fungus
The endophytic fungi isolated from the leaves of *M. correia* were identified based on the morphological and molecular method. The morphological identification based on their conidial morphology and spores under microscope. Fungal molecular identification was carried by internal transcribed spacer (ITS) amplification. The genomic DNA of endophytic fungi was extracted according to the method of Miller *et al* [16]. Fungal ITS rDNA regions were amplified by the universal ITS primers ITS1 (5′-TCCGTAGGT-GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3') with the following parameters: 1 min initial denaturation at 94°C followed by

30 cycles of 30-s denaturation at 94°C, 1-min primer annealing at 57°C, 90-s extension at 72°C, and a final extension period of 10 min at 72°C (Nakahama *et al*., 1973) [17]. The amplified product was electrophorized on 1.5% agarose gel and was sequenced by outsourcing (Using sanger' sdideoxynucleotide chain termination method) at Eurofins genomics, Bangalore. The sequences were compared with the GenBank database using the NCBI Blast program (http://www.ncbi.nlm.nih.gov/blast/). Sequence data were deposited in the GenBank and Accession Number were obtained. Multiple sequence alignment was performed using Clustal X and a phylogenetic tree was constructed by the Neighbor-joining method (Saitou and Nei, 1987) [18] using MEGA X software (Kumar *et al*. 2018) [19].

2.3 Extraction of bioactive compounds

The endophytic fungal isolates were inoculated into 250ml Erlenmeyer flasks containing 100 ml Czapek'sdox (CDB) broth and incubated at 25°C and 150 rpm for 15 days. The crude fermentation broth was separated through Whatman no.1 filter paper and the liquid filtrate was extracted with an equal volume of ethyl acetate (Strobe *et al*., 1996) [20]. The ethyl acetate fractions were evaporated under reduced pressure at 32°C using rotary vacuum evaporator. The solid ethyl acetate extracts were dissolved in 1ml of methanol for further analysis.

2.4 Evaluation of Antimicrobial activity

The antimicrobial assay was carried out by the disk diffusion technique. The microorganisms used in this experiment were screened against human pathogenic bacteria *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Bacillus subtilis and Klebsiella pneumonia.* The respective cultures were grown on nutrient broth medium for 12 h. For disc diffusion assay, 100µl of overnight grown bacterial culture was spread over the nutrient agar medium. The concentrated bioactive compound from the culture filtrate of endophytic fungus was placed (100µl) over sterile disc. Streptomycin/Ampicillin disc served as positive control and for negative control methanol was used. The plates were incubated overnight at 37ºC and the results were recorded as an inhibition zone in mm.

2.5 Chromatographic analysis

GC-MS analysis

The crude extracts of *C. gloeosporioides* was analyzed using Agilent technologies 6890 instrument. An inlet of 0.75 mm I.D., which improves the GC resolution, was used. The carrier gas was helium (1 mL.min–1) and the injector temperature was 260°C. The analytes were separated on an HP-5MS 30.0m x 250µm column (Supelco, Inc., Bellefonte, USA), kept at 60°C for 2 minutes and then ramped to 300°C at 10°C/min and held at the final temperature for 6 minutes. The transfer line was kept at 240°C and the ion source was held at 240°C. Mass spectra were measured at 70 eV and collected at the rate of 1 scan/second over an m/z range of 40 to 600. Chromatographic retention indices of separated compounds were calculated relative to a C8-C22 n-alkanes mixture.

3. Results and Discussion

3.1 Isolation and identification of fungal endophytes

Two endophytic fungi were isolated from the healthy leaves of *Morinda correia*. The isolated fungus was identified as *Colletotrichum gloeosporioides*, *Alternaria tenuissima* morphologicaly by Dr. Prameela Devi, IARI, New Delhi, India and allocated to the ITCC number (Table 1). Molecular identification was done by the analysis of the rDNA sequence and the sequence were deposited in the GenBank and obtained accession numbers (Table1).

3.2 Phylogenetic analysis

ITS sequences of the endophytic fungi were compared with the available data in NCBI. Multiple Sequence Alignment was performed using Clustal X software which shows 100% sequence similarity (Fig. 1a, b) towards selected species. The evolutionary phylogenetic tree was constructed for two isolates using the Neighbor-joining method (Fig 2a, b). The evolutionary distances were computed using the Maximum Composite Likelihood Method (Tamura *et al*., 2004) [21] and are in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985) [22]. The analysis involved 10 nucleotide sequences. Evolutionary profile was conducted in MEGA X.

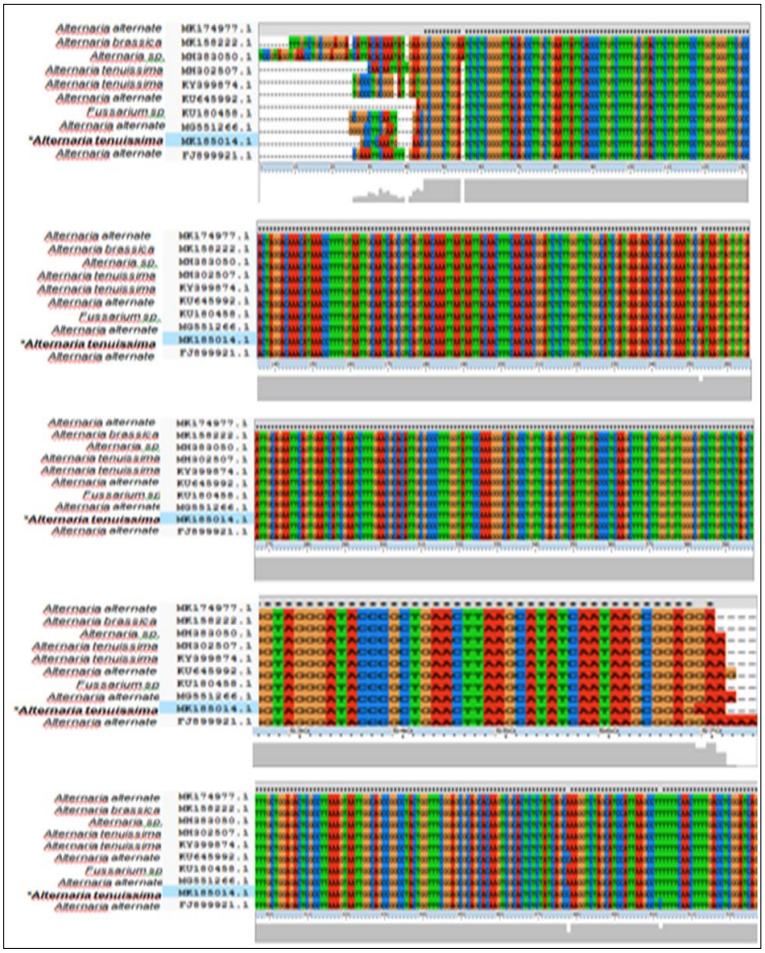

Fig 1a: Multiple sequence alignment (MSA) of *Alternaria tenuissima**

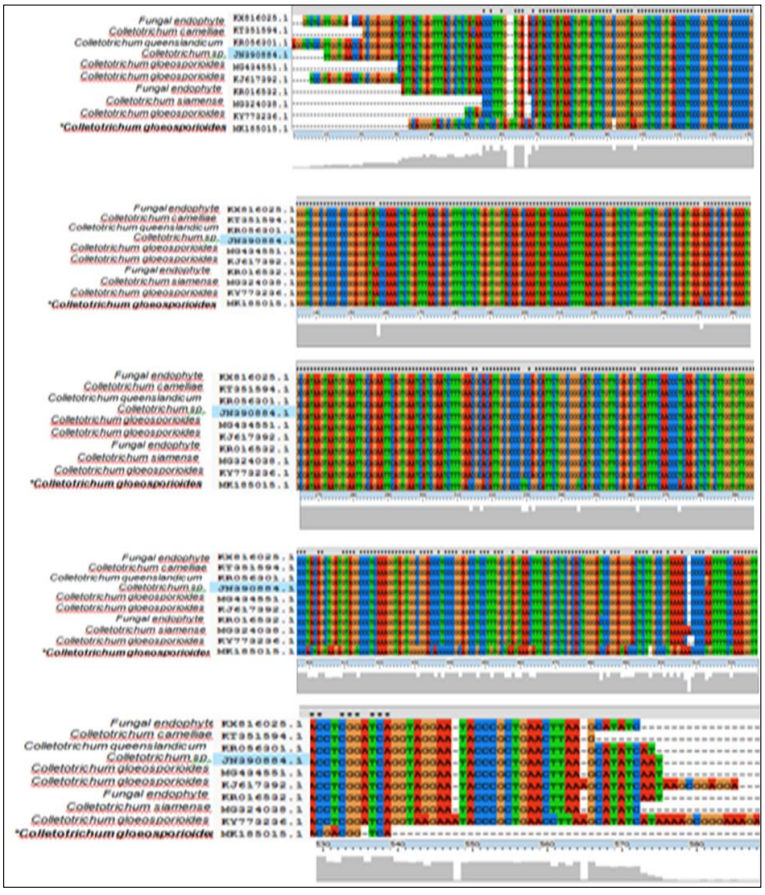

Fig 1b: Multiple sequence alignment (MSA) of Colletotrichum *gleosporioides**

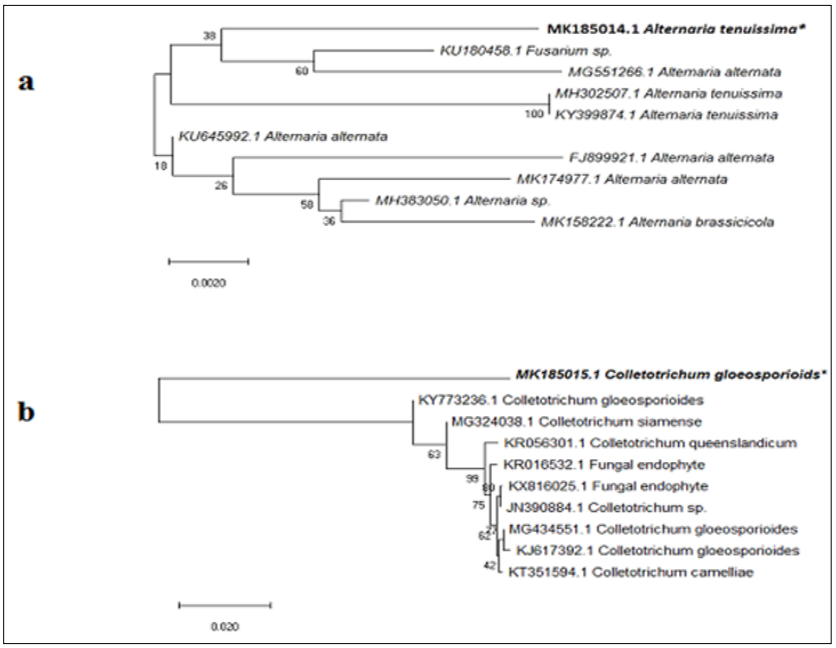

Fig 2: Phylogenetic tree (Neibour-joining) of the ITS sequence of (a) *A. tenuissima** (b) *C. gleosporioides**

3.3 Antimicrobial activity

Ethyl acetate extracts obtained from *Colletotrichum gloeosporioides*, *Alternaria tenuissima* exhibited antimicrobial property under *in vitro* condition (Fig. 3a, b). However, extracts obtained from *C.gloeosporioides* revealed high antimicrobial activity when compared to *A. tenuissima* inhibiting the test bacteria (Table 2a, b). Maximum antibacterial activity was observed against *S.aureus* (36mm), followed by *B. subtilis* (35 mm), *K. pneumonia* (34mm), *S. typhi* (33 mm) and *E. coli* (32 mm). The ethyl acetate extract showed higher antibacterial activity than the standard antibiotics. Various researchers reported the antimicrobial, antifungal, antiviral properties of the extracts of endophytic microorganisms (Gunatilaka 2009; Fisher *et al*., 1986; Wiyakrutta *et al*., 2004; Teles *et al*., 2006) [23, 24, 25, 26].

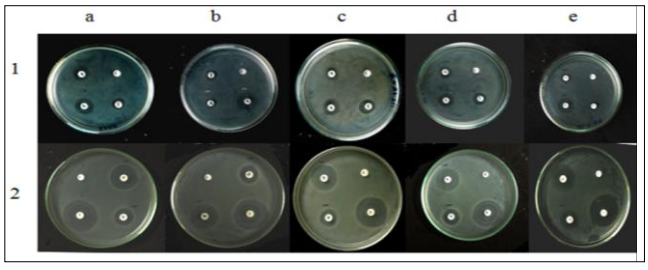

Fig 3: Antibacterial activity 1) *A. tenuissima* 2) *C.gloeosporioides.* a) *E.coli* b) *K.pneumonia* c) *B.subtilis* d) *S.typhi* e) *S.aureus*. C- Negative control, A – Ampicillin, S – Streptomycin, T- Test

3.4 GC-MS analysis

Gas chromatography–mass spectrometry (GC–MS) is a useful tool for analysis of a wide range of relatively volatile compounds, and the technique has been widely applied in Medical, biological, and food research. The ethyl acetate extract of *C. gloeosporioides* exhibited 22 major peaks in GC analysis (Table 3). The GC-MS chromatogram is illustrated in Fig 6. Tetradecane, Hexadecane, Tetratetracontane, Heptacosane, Eicosane, Dibutyl phthalate, Heneicosane, Hepatadecanoic acid was the major compound, which is the active principle compound in *C. gloeosporioides*. Tetradecane has nematicidal activity against *Bursaphelenchus xylophilus* (Seo *et al*., 2010) [27]. Hexadecane has been reported to have antibacterial activity in the crude extract of *Spirulina platensis* (Kumar *et al*., 2011) [28]. Tetratetracontaneis an antibacterial compound (Gumgumjee *et al*., 2015) [29] and is known as an antioxidant and cytoprotective agent (Mallick and Dighe, 2014) [30]. Hepatadecanoic acid has been reported to bear antioxidant activity (Henry *et al*., 2002) [31]. Heneicosane was reported to possess anti-insecticidal property and acts as an ovulation repellent in high doses to *Aedesaegypti* females (Seenivasagan *et al*., 2009) [32]. Eicosane (Karanja *et al*., 2010; Nandhini *et al*., 2015) [33, 34] and Dibutyl phthalate (Roy *et al*., 2006) [35] were reported to be antifungal compounds. Heptacosane was reported to have anti-insecticidal activity (Atawodi *et al*., 2009) [36]. Derivatives of decane shown potential anti-inflammatory activity (Mullen *et al*., 1987) [37]. The GC-MS chromatographic analysis of the ethyl acetate extracts divulge the presence several of bioactive compounds. Therefore this type of GC-MS analysis is the first step in understanding the nature of the active principles in this medical plant and this type of scrutiny will be useful for further detailed study. Further investigation may lead to the isolation of bio-active compounds, and their structural elucidation and screening of pharmacological activity may be useful for further drug development.

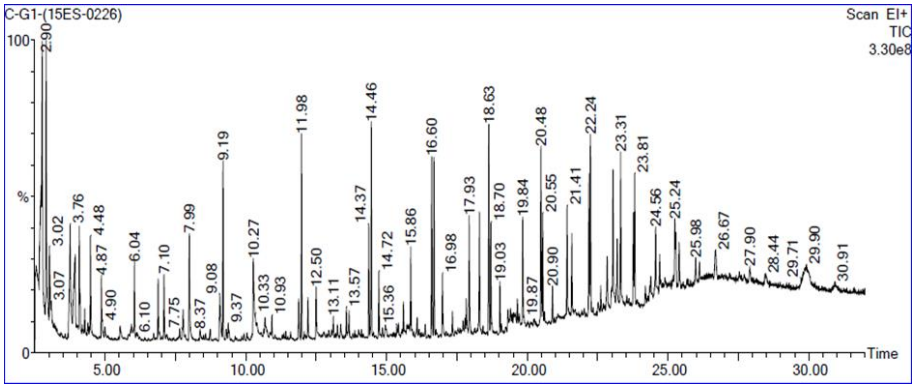

Fig 4: GC-MS spectrum of ethyl acetate extract of endophytic fungus *C.gloeosporioides*

Table 1: Identification of endophytic fungi isolated from M. *correia*

Fungal name	ITCC.NO	Genbank Accession No.	Percentage of similarity
Alternaria tenuissima	9840.15	MK185014	100
Colletotrichum gloeosporioides	9839.15	MK185015	100

Table 2: Evaluation of antibacterial activity of fungal extracts

Test bacteria	Zone of inhibition (mm)						
	NC	Fungal extracts					
		AT			*CG*		
		Std		Ex	Std		Ex
		Amp	Str		Amp	Str	
E.coli	0	16	20	15	20	23	32
K.pneumonia	0	13	22	14	16	25	34
B.subtilis	0	16	19	18	18	22	35
S. typhi	0	14	18	16	19	24	33
S.aureus	0	15	21	16	15	26	36

AT - Alternaria tenuissima, CG - Colletotrichum gloeosporioides, NC – Negative control, Std – Standard, Amp - Ampicillin, Str – Streptomycin, Ex – Extract.

Table 3: List of compounds identified by GC-MS from the extract of *C. gloeosporioides*

R.T	Compound	M.W	Formula
2.73	Propanoic acid,2 methyl	88	C4H8O2
2.904	Butanediol,[r-(r*,r*)]	90	C4H10O2
3.759	Propanedioic acid	104	C3H4O4
3.994	Butanoic acid,2-methyl	102	C5H10O2
7.991	1,3-butanediol,diacetate	174	C8H14O4
9.186	Undecane,4,6-dimethyl-	184	C13H28
10.266	Dl-mevalonic acid lactone	130	C6H10O3
11.977	Tetradecane	198	C14H30
14.368	Tetradecanol	214	C14H30O
14.458	Heneicosane	296	C21H44
16.604	Hexadecanoic acid	284	C18H36O
16.684	Eicosane	282	C20H42
17.935	2-hexadecanol acetat e	284	C18H36O2
18.295	Dibutyl phthalate	278	C16H22O4
18.630	Hexadecanoic acid, ethyl ester	284	C18H36O2
20.481	Methyl 17-methyl-octodecanote	312	C20H40O2
22.186	Hepatadecanoic acid, ethyl ester	298	C19H38O2
22.241	Tetratetracontane	618	C44H90
23.042	Hepatacosane	380	C27H56
23.312	1,2-benezene dicarboxylic acid, mono (2-ethyl)ester	278	C16H22O4
23.812	Tetratriacontane	478	C34H70
24.557	Octadecane,3-ethyl-5-(2ethylbutyl)	366	C26H54

4. Conclusion

Endophytic microorganisms are a reliable source of bioactive and chemically novel compounds with the ability for utilization in a wide variety of medical, agriculture and industrial areas. This study, demonstrates the isolated endophytic fungi from *M. correia* for the control of human pathogens. The ethyl acetate extract of the endophytic fungus *C. gloeosporioids* displayed greater antimicrobial activity against all the tested bacteria which is more than the positive control. All these results indicate that *C. gloeosporioids* can be used as an alternate source to obtain novel bioactive metabolites.

5. Acknowledgement

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6. Conflict of interest

The authors declare that they do not have any conflict of interest.

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