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Anti-cancer potentials of endophytic fungi isolated from *Enicostemma axillare* and *Ormocarpum cochinchinense*

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Abstract
In the present study, anti-cancer activity of the endophytic fungi isolated from two medicinal plants, *Enicostemmaaxillare* and *Ormocarpumcochinchinense* was examined. Four endophytic fungi were isolated and identified based on morphological characteristics and microscopic structures. The extracts were screened for cytotoxicity against MCF-7 cell lines by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5Diphenyltetrazolium Bromide) assay. The fungus *Phialophora* isolated from *Enicostemmaaxillare* showed the highest cytotoxicity. Further, the fungal extract EAR2 was purified using preparative TLC. GC-MS (Gas Chromatography-Mass Spectrometry) analysis was carried out to identify the compounds present in the purified extract. Isopropyl myristate was identified as the major compound.

Keywords: endophytic, *Enicostemmaaxillare*, *Ormocarpumcochinchinense*, anti-cancer, MTT, isopropyl myristate

Introduction
Human health around the world is threatened by cancer, AIDS, and various other infectious diseases (Guo *et al*., 2008, Wang *et al*., 2008) [7, 31]. There is a constant need for the discovery and development of anti-cancer drugs because of a few restrictions of existing drugs such as low tumor selectivity, narrow therapeutic index and development of multidrug resistance (Coates *et al*. 1983, Persidis 1999) [3, 20]. The medicinal plants are being considered for control of malignancies owing to their low levels of cytotoxicity and drug resistance phenomenon (Mbaveng *et al*., 2011) [14]. For instance, plant derived compounds such as camptothecin and taxol have shown to play a vital role in cancer treatment (Srivastava *et al*., 2005) [26].
Endophytes are micro-organisms that colonize living plants at certainor at all stages in their life, without harming the host. Surface saprophytes, latent pathogens and mycorrhizal fungi are some of the micro-organisms which inhabit the internal tissues at certain part of their lifecycle (Petrini & Fisher, 1990) [21]. While existing in the plant tissues, some of the endophytic fungi are proven to be producers of same or similar secondary metabolites as their hosts with interesting biological activities (Tan & Zou, 2001) [28]. Owing to their enormous production of bioactive metabolites, they have been considered to be a potential anticancer source (Kharwar *et al*., 2011) [11].
Enicostemmaaxillare belongs to the family Gentianaceae. It has been used in the Indian system of medicine to treat various skin diseases, helminthiasis (Warrier *et al*., 1995) [33] and tumors (Mudaliar, 2002) [17]. The anticancer potential of the plant has been investigated in rodent and cellular models (Kavimani and Manisenthilkumar, 2000) [10]. The methanolic extract has been proven to possess significant inhibitory effect on the growth of cancer cells (Krishna and Mohandass, 2014) [29]. The plant is known to be a blood purifier and has been used in the treatment of Dermatopathy and Venereal infections (Pandikumar *et al*., 2011) [18].
Ormocarpumcochinchinense is a medicinalshrub belonging to the family Fabaceae (Shanthi P, 2008) [24]. It is locally known as Elumbotti or Kattumoringai in Tamil. The root is utilized as a tonic, stimulant and used in treatment of lumbago and paralysis. The leaves are included in formulations used for setting bone fractures and fornervous pain (Dinesh kumar M *et al*., 2013) [5].
In the present study, anti-cancer potentials of the endophytic fungi isolated from *Enicostemmaaxillare* and *Ormocarpumcochinchinense* was evaluated by MTT assay. Further, the fungus exhibiting the highest cytotoxic activity was purified by preparative TLC and cytotoxicity of the purified extract was examined.

Materials and Methods

Isolation of endophytic fungi

Samples of fresh and uninfected leaves of *Enicostemmaaxillare*and *Ormocarpumcochinchinense*were collected and processed. The leaves were washed in running tap water until they are cleaned thoroughly and cut into small square pieces using sterile scalpel. Thesamples were surface sterilized with 70 % ethanol for 5 s, sodium hypochlorite for 90 s, 0.1% mercuric chloride for 240 s, and finally rinsed with sterile distilled water. Surface sterilized samples were blotted dry, trimmed using sterile blade and placed on potato dextrose agar (PDA) medium amended with 100 mg/L ampicillin to avoid bacterial growth. The plates were incubated at 25ºC ± 2º0C and observed for the growth of endophytic fungal colonies. The colonies emerging from the plant segments were subculturedon to fresh PDA plates to obtain pure cultures (Singh *et al*., 2012) [25].

Identification of the endophyticfungi

The isolated endophytic fungispecies were identified according to their microscopic and macroscopic structures. The taxa were assigned to different genera as specified by taxonomists. The fungal isolates that failed to sporulate were classified under the category of mycelia sterilia (Wang Y *et al*., 2012; Ellis MB, 1971; Barnett HL and Hunter BB 1998; Ainsworth GC *et al*., 1973) [32, 6, 2, 1].

Preparation of fungal organic extract

Few discs from the edges of the growing cultures of isolated fungi were inoculated in 500 ml Erlenmeyer flask containing 300 ml of Potato Dextrose Broth (PDB)(20% Potato infusion, 2% Dextrose and 2% agar) and incubated in dark at 25°C±2°C for 21 days under static conditions. The filtered broth was extracted with twovolumes (v/v) of ethyl acetate. The extract was dried over anhydrous sodium sulfate and then evaporated under vacuum in a rotary evaporator. The crude extracts were then dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at 4°C as stock solution for phytochemical analysis and anticancer bioassays (Von Arx, 1978) [30].

Human cancer cell lines and culture conditions

MCF-7 cells were procured from National Centre for Cell Sciences (NCCS), Pune, cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 U/ ml of streptomycin in a 5% CO_2 atmosphere at 37°C.

Invitro cytotoxic activity of crude fungal extracts using MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5Diphenylte trazolium Bromide] assay

The cytotoxic effect of fungal crude extracts on MCF-7 cell line was assayed using MTT assay. Briefly, the cells were seeded in 96 well plates at a density of 1 X 10^4 cells/well for 24 h. Subsequently, the fungal crude extracts dissolved in 0.4 % DMSO, were added at different concentrations and incubated for 24 h. Post incubation, MTT reagent (5 mg/ml) was added and incubated for 4 hin dark. The blue MTT formozan precipitate formed was solubilized in DMSO and measured spectrophotometrically at 570 nm in an ELISA plate reader scanning spectrophotometer. Cells grown in culture media alone or with appropriate concentrations of DMSO were used as controls. Absorbance data were converted into % cell inhibition according to the following equation:

Cell inhibition = {(Abs. value of control- Abs. value of sample)/Abs value of control} x 100%

The correlation of cell death and extract concentration was then analyzed by using a line regression test. The extract concentration required to inhibit cancer cell growth by 50% (IC50) was then calculated from the dose-response curve (Mossman T., 1983, Lin L and Hwang PL, 1991; Hasan AEZ *et al*., 2014) [16, 13, 8].

Preparative-plates Chromatography

Chromatography was performed on 50 mm × 100 mm glass plates pre-coated with 2 mm layers of silica gel Si 60 HF254. Samples were applied to the edge of the layer by use of the mobile phase distributor of the DS chamber. Plates were developed face-down, to a distance of 8 cm, in a horizontal Teflon DS chamber after conditioning with mobile phase vapour for 15 min. After development, the mobile phase was evaporated to dryness and the band matching with the Rf value was scraped off, resuspended in solvent and centrifuged. The supernatant was allowed to concentrate by heating it at 30ºC in a hot lid (Jóźwiak GW and Hajnos MW, 2007) [9].

Gas Chromatography-Mass Spectrometry analysis

The fungal extract was subjected to GC MS analysis to identify the bioactive compound. The sample was analysed in GC Clarus 500 Perkin Elmer by using software Turbo mass 5.2 equipped with mass detector Turbo mass gold Perkin Elmer. 2µl sample was introduced via an allglassinjector working in the split mode, with He as the carrier gas with a linear velocity of 32 cm/s. The HP-5fused silica capillary column (Length – 30 m; Film thickness- 25 µm I.D - 0.2 mm) was used. The identification of components was accomplished using computer searches in NIST version 2005 (Devi NN and Singh MS, 2012) [4].

Results

A total of 30 endophytes were isolated from the healthy leaves of *Enicostemmaaxillare* and *Ormocarpumcochinchinense.* The isolated endophytic fungi were identified based on the culture characteristics, morphology and growth. The identified isolates were *Phialophora, Penicilliuim and Aspergillus*, and the remaining isolates which failed to sporulate were categorized as mycelia sterilia.

All the isolates were subjected to cytotoxicity analysis by MTT assay. The percentage cytotoxicity values of the extracts along with the positive control are shown in Table 1. The fungal isolate EAR2 showed the maximum percentage cytotoxicity of 64.54% at a concentration of 125µg. Therefore, the extract EAR2 was purified using preparative TLC and the purified extract was analyzed for the presence of bioactive compounds by GC-MS. The analysis indicated that *Phialophora* from *Enicostemmaaxillare* produced bioactive compounds, which were identified based on the NIST database by virtue of comparisons made of the actual mass spectral data acquired shown in Figure 5. The chromatogram predicted the presence ofisopropyl myristate at a retention time of 20.26 min.

Table 1: The percentagecytotoxicity values of the fungal extracts and positive control are tabulated.

Concentration (µg/mL)	Cytotoxicity %				
	MCF7				
	EAR2	EAR1B	OSLM1A	OSLM1B	Positive Control
25	42.53	38.91	38.50	38.98	47.47
50	44.27	43.03	41.10	41.66	51.01
75	46.46	46.23	43.70	45.99	56.72
100	60.98	50.86	49.67	52.12	68.52
125	64.54	53.60	53.87	54.83	75.34

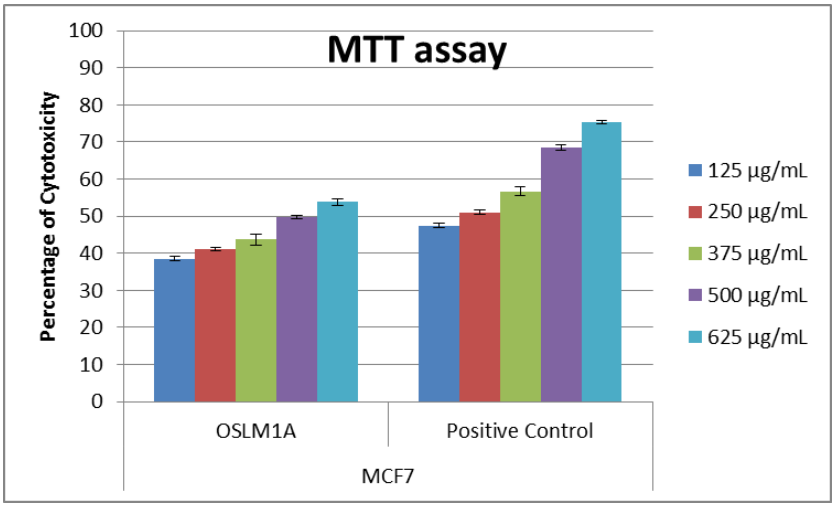

Fig 1: The percentage of cytotoxicity of the extract OSLM1A against MCF7 cells.

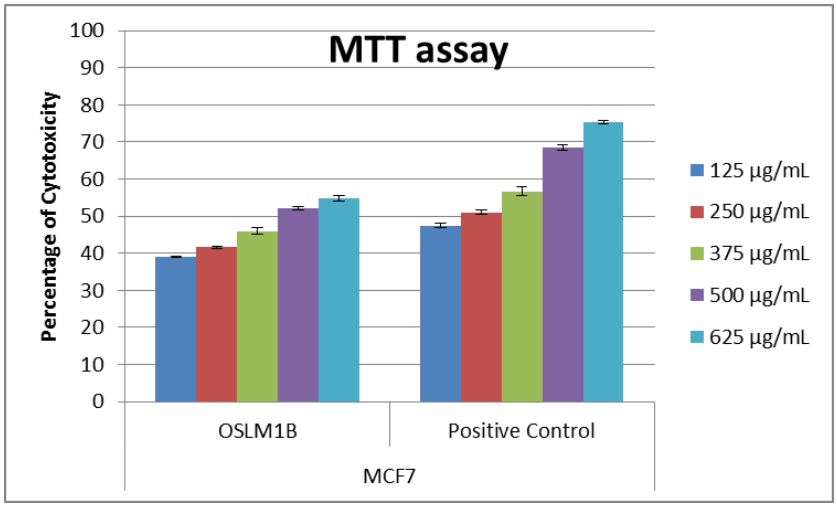

Fig 2: The percentage of cytotoxicity of the extract OSLM1B against MCF7 cells.

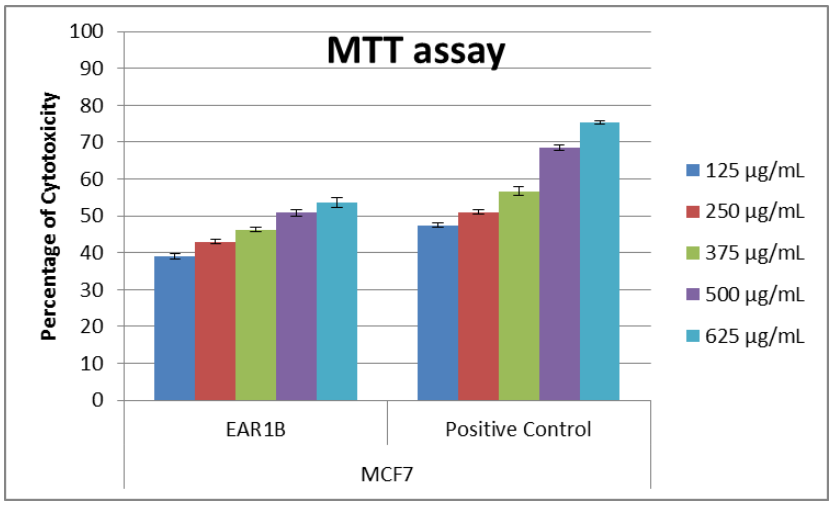

Fig 3: The percentage of cytotoxicity of the extract EAR1B against MCF7 cells.

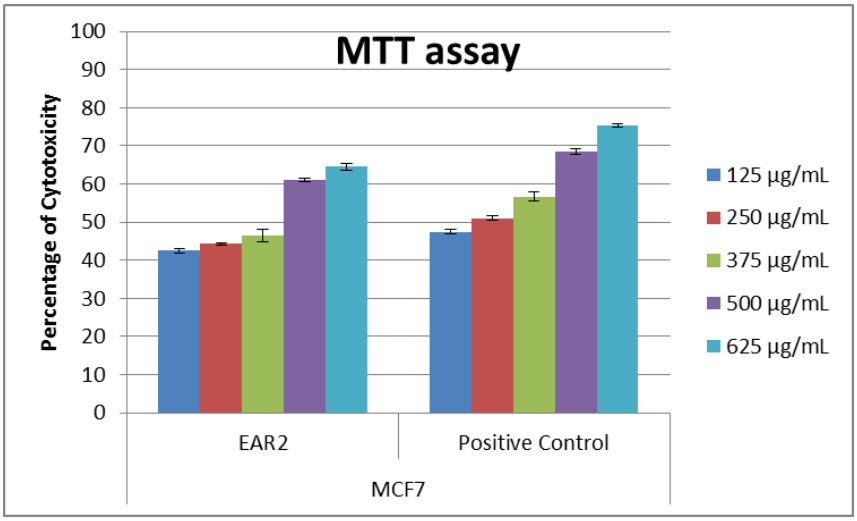

Fig 4: The percentage of cytotoxicity of the extract EAR2 against MCF7 cells.

Table 2: The IC50 value of the fungal extracts in MCF7 cells (in µg/mL).

Extract	EAR2	EAR1B	OSLM1B	OSLM1A
IC 50 (µg/mL)	338.62	491.27	472.78	523.18

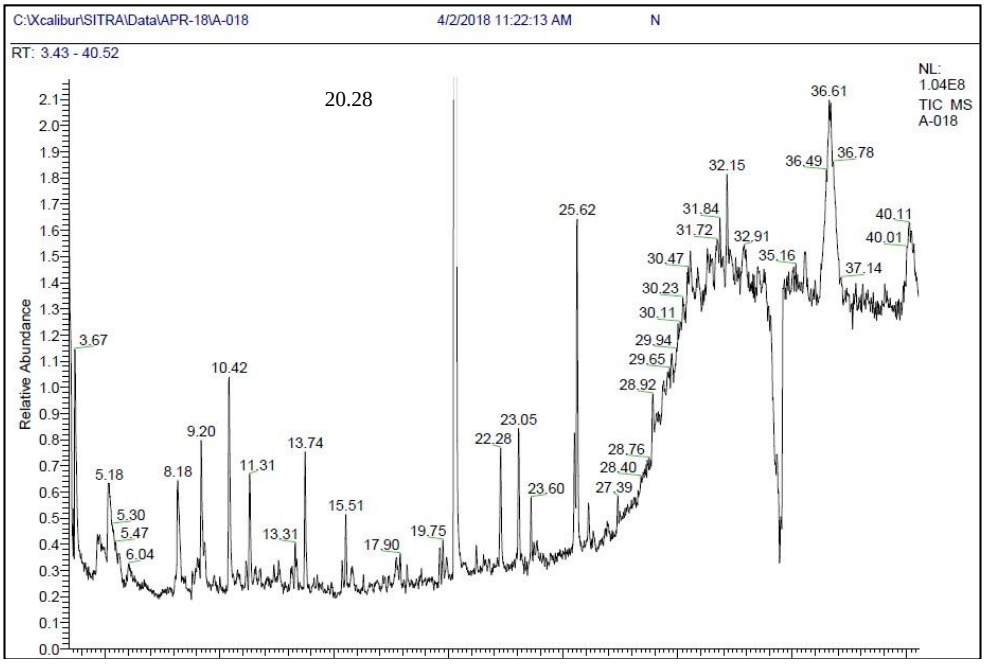

Fig 5: GC-MS chromatogram of EAR2 isolated from *Enicostemmaaxillare*.

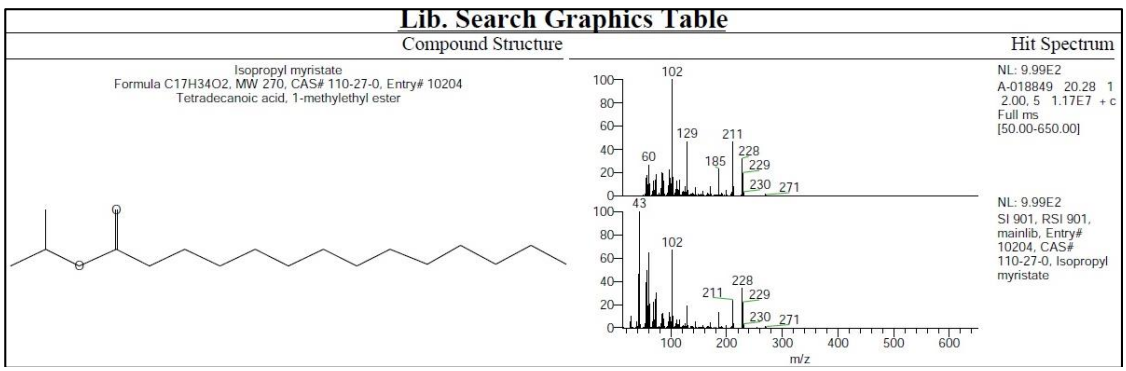

Fig 6: Structure of the compound detected by GC-MS at 20.28 min.

Discussion

Fungal endophytes have gained special attention due to their potential to produce metabolites which can prove to be of pharmacological interest and the role that they play in regulating the ecological systems at different trophic levels. Endophytic fungi from plants are well known sources of

bioactive secondary metabolites (Schulz *et al*, 2002) [23]. They are reported to be reservoirs of novel compounds with idiosyncratic bioactivities (Tan RX *et al*, 2001) [28]. The secondary metabolites of endophytic organisms include alkaloids, steroids, terpenoids, quinones, lignans, phenols, flavonoids, aliphatic compounds, etc. The phytochemicals within the endophytes could be a potential source for medicinal and industrial use and they are also considered to be a potential source of precursors in the development of synthetic drugs (Sadananda *et al*, 2011) [22]. There is an increasing effort to characterize and identify the endophytic fungi isolated from medicinal plants.

With this rationale, two medicinal plants*Enicostemmaaxillare* and *Ormocarpumcochinchinense* were chosen for the isolation of endophytic fungi from leaves. Till date, there has not been much investigation carried out on the endophytic fungi of the above mentioned plants.It is known that endophytic fungi are a rich source of anticancer drugs. Some endophytic fungi possess the ability to produce important anticancer drugs or their derivatives, which have been used clinically. Screening and evaluating novel anticancer compounds from endophytic fungi will be an effective and time saving alternative to other sources (Kuriakose *et al*, 2014) [12]. In the present study, the cytotoxic activity of fungal extracts was investigated on Human breast adenocarcinoma cells (MCF-7) by using MTT assay. The crude extract of the endophyte, *Phialophora*isolated from *Enicostemmaaxillare* was proven to be cytotoxic against MCF-7 cells among other extracts. It was found that the most ubiquitous phylum of fungi is Ascomycota, which is among the most prevalent group of eukaryotes worldwide (Zare R *et al*., 2007; Pereiro M *et al*., 2004) [34, 19] The results indicate that the fungal extract EAR2is an excellent candidate for consideration in anticancer drug formulation. Further, GCMS analysis revealed the presence of many bioactive compounds. The major compound identified was isopropyl myristate, an ester of isopropyl alcohol and myristic acid. Wheat germ agglutinin-conjugated PLGA nanoparticles containing isopropyl myristate and paclitaxel has proven to show enhanced anti-proliferative activity through Isopropyl myristate facilitated fast release of paclitaxel from the Nanoparticles to arrest the cell growth, which suggest that isopropyl myristate might play an important role in the treatment of cancer (Mo Y and Lim LY, 2005) [15].

Conclusion

In the present study, anti-cancer activity of endophytic fungi isolated from *Enicostemmaaxillare* and *Ormocarpumcochinchinense* was evaluated by MTT assay. The isolated fungal species were identified based on the culture morphology. The fungus *Phialophora* showed the highest cytotoxic activity. Further, the fungal extract showing the highest cytotoxic activity was subjected to preparative TLC for purification. The purified compound was analyzed by GC-MS. Isopropyl myristate was identified as the major compound. Further studies have to be performed to evaluate the apoptotic induced death of the cancer cell lines.

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