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Cytotoxicity effects of amyrin produced by the endophytic fungi, *Colletotrichum gloeosporiodes*

Abstract

Colletotrichum gloeosporiodes was isolated as the true endophytic fungi from the rare and endangered tree *Cynometra travancorica*, occurring in Western Ghats region of India. The endophyte was cultured for 40 days in Potato Dextrose Medium for the screening of secondary metabolites. The compounds were isolated and purified by thin layer and column chromatography. Structural characterisation was done by IR, NMR and Mass spectrum. The compound was identified to be beta amyrin.

Keywords: *Cyanometra travancorica*, endophytic fungi, *Colletotrichum gloeosporiodes*, beta-amyrin

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Introduction

Endophytic fungi are a precious resource of rare and valuable compounds which possess broad range of therapeutic properties. After the discovery of taxol from the endophytic fungi, *Taxomyces andreanae,* endophytic researches have gained importance. Tropical endophytes were target for study in recent years but few studies are addressed the problem about the therapeutic potentials.[1] There are evidences suggesting that endophytes evolved directly from plant pathogenic fungi.[2,3] These organisms dwell in novel biotopes and produce novel compounds with therapeutic potentials. *Cynometra travancorica* is a legume of fabaceae family. It belongs to the allied taxa of *Saraca asoka* which is well known for medicinal properties.[4] *C.travancorica* is an endangered tree found endemic to Western Ghats. The bark of the tree is used by tribals for uterine disorders. *C.travancorica* is reported to have antimutagenic potential.[5] Considering the therapeutic potentials and as evergreen forests of Western Ghats are a treasure house of rare endophytic fungi, we selected the Cynometra species for our present study.

Materials and methods

Chemicals

Oat meal agar was obtained from Himedia. Protease inhibitor cocktail was purchased from Sigma. TRIZOL reagent, Eagle's minimum essential medium (MEM) was obtained from HiMedia (India).

Sample collection

The material was collected from 4 different evergreen forest–Shendurney (8 8727 N, 77 1634 E), Siruvani (7°10 N, 10°55' E), Thamarassery Ghat (11°26' N, 75 53E) and Vellanimala (10° 25N and 76° 30E) of Kerala. The collection was made in 3 different season, pre monsoon, monsoon, post monsoon. Three samples per location per season were collected for the analysis.

Isolation and characterisation of endophytes

The explants were surface sterilized with 75% ethanol for 60 seconds. The tissues were rinsed in sterile distilled water 3 times and allowed to surface dry in sterile conditions.[6] Oat meal agar was found to give optimum growth for fungal isolation (rapid growth and more number of colonies). Five segments of leaves with 0.5 cm diameter were evenly placed in petri dishes containing oat meal agar with streptopenicillin, to suppress bacterial growth and incubated for 20 days with 12 hrs of light followed by 12 hrs of dark cycles at room temperature. A total of 40 segments were analysed per sample. The explants were monitored everyday to check growth of endophytic fungal colonies. The hyphal tips when grew out from leaf segments were isolated, sub cultured and stored at -4°C on oat meal agar slants for preservation. The cultures which were constantly yielded in all the collections were recorded as the true endophytic fungi. The isolates were maintained by routine sub culturing. Identification was done by partial sequencing of genomic DNA.

Isolation of secondary metabolites from endophytes

The endophytic fungi were cultured in bulk quantities in Potato dextrose broth at room temperature. The cultures were incubated for 40 days. The broth after removal of fungal mycelium was filtered, concentrated by heating on a boiling water bath. The concentrated broth was treated with five different solvents, petroleum ether, dichloromethane, ethyl acetate, methanol and water batch wise in a separating funnel. The extracts were separated using Thin Layer chromatography (TLC) followed by column chromatography using suitable solvents.[7] The isolated compounds were characterised by IR, NMR and LC-MS. IR was done at STIC facility, Cochin University of Science and Technology, Kalamassery, Ernakulam, Kerala, India, NMR and LC-MS analysis were done at SAIF, IIT, Madras, Chennai, India.

Resazurin reduction assay

Cells were seeded in a 96 well plate at a density of 5000 per well and grown overnight. Cells were treated with increasing concentration of terpenoids ranging from (2-10 mg) in complete DMEM from the mother stock. Analysis of cytotoxicity after 48h treatment was determined by resazurin reduction assay with slight modifications.

At a concentration of 0.1 mg/ml, resazurin dye was added on to the media, incubated for 3 h, for the reduction of blue dye resazurin to pink resorufin which is read at 570-590 nm. The data were analysed as percent control. IC_{50} was obtained by determining the concentration of compounds resulting in 50% inhibition of viability in colorectal cancer cells after 48h.

Results and discussion

Twelve pure cultures of endophytic fungi were obtained from the leaves of *Cynometra travancorica.* The leaf cultures constantly yielded an organism which was identified a *C.gloeosporiodes* by molecular methods. The sequence has been submitted in gene bank with the accession number KM823608 (Figure 1). Koch's postulates were confirmed (Figure 2). From the IR spectrum the peaks, 2042 cm^{-1}, 2947 cm^{-1} and 3404 cm^{-1} indicate the presence of terpenoid. The peaks show presence of aliphatic compound. NMR spectrum reveals the presence of aliphatic compounds. The LC-MS peak gives the molecular mass of the compound. From the LC-MS spectrum the mass of the compound was determined to be 201.05 kDa which is of amyrin (Figure 3–5). The terpenoids were cytotoxic to the SW620 cells in a dose dependent manner after 48h of incubation. The IC_{50} value was found to be 4 micrograms Table 1.

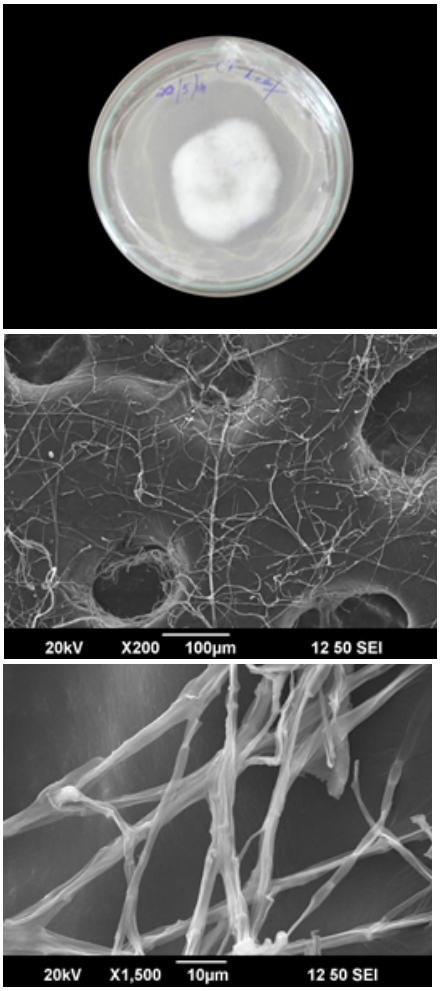

Figure 1 a. *Colletotrichum gloeosporiodes* characterised as true endophyte from *Cyanometra travancorica.* b. Scanning electron microscope image of mycelium of *C.gloeosporiode*. c. Scanning electron microscope image of hyphae of *C.gloeosporiodes*.

Figure 2 Confirmation of Koch's postulates to affirm the pathogenicity of the strain.

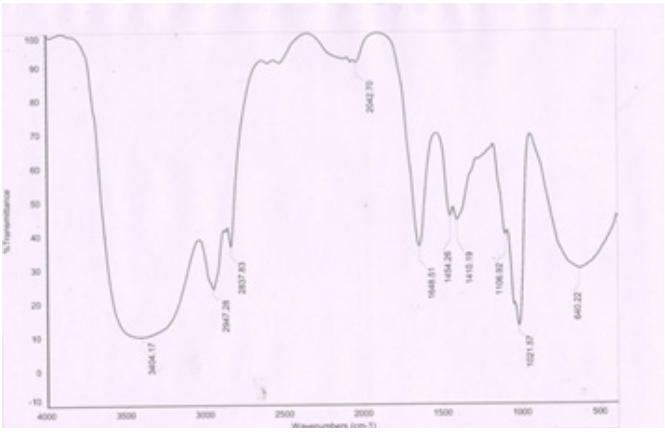

Figure 3 IR spectrum of Ct.

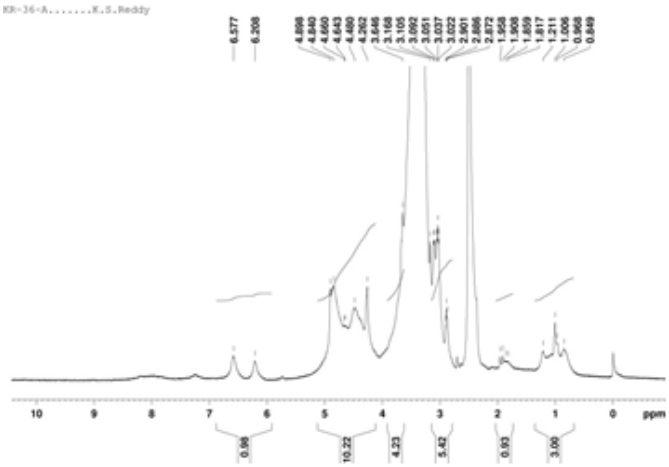

Figure 4a HNMR spectrum of Ct.

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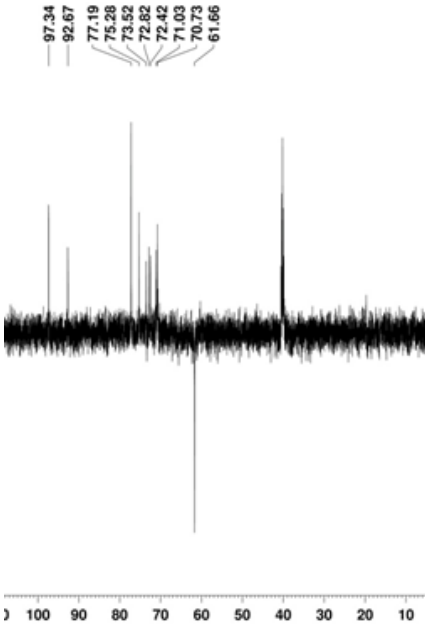

Figure 4b CNMR of Ct.[13]

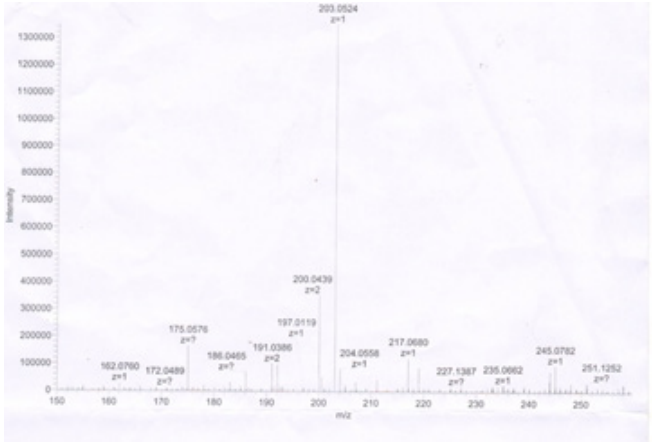

Figure 5 LC-MS of Ct.

Colletotrichum gloeosporioides is a well known pathogen. The organism interacts with numerous plant species blatantly as symptomatic pathogens and cryptically as asymptomatic pathogens. The genus *Colletotrichum* (Sordarimycetes, Ascomycota) comprises~600 species.[8] As per ARS fungal databases, it attacks over 3,200 species of monocot and dicot plants. The infection strategy of this organism is a multistage hemibiotrophy.[9] *Colletotrichum* gloeospoirioides has been reported to produce taxol.[10] The establishment of endophytic association with plants are interesting, the initial steps being the same–recognition, germination and penetration and then a quiescence stage is developed. There may be some mechanism to avoid recognition also. For example a gene has been cloned from *Colletotrichum gloeosporioides* which is switched on during the initial phase of colonisation and switched off later during the neurotropic phase.[11] This gene encodes a glycoprotein that resembles plant cell wall proteins which is believed to coat the hyphae that the plant is unable to recognise as alien (Figure 6).

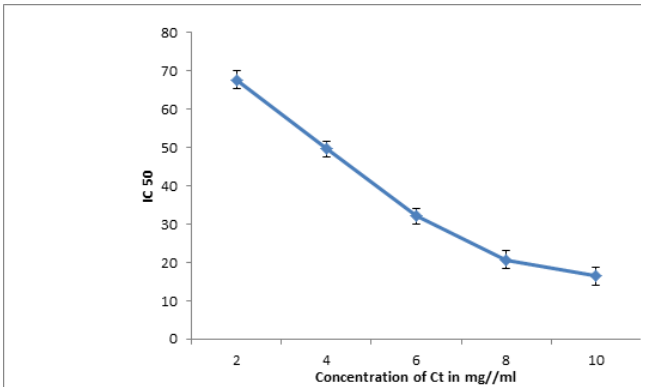

Figure 6 Resazurin assay showing IC_{50} of Ct in SW620 cells.

Terpenoids are naturally occurring compounds made up of isoprene units, which are the largest group of natural compounds (60%). Productions of secondary metabolites are one of the enthralling features of the fungal endophytes. These compounds are produced by the fungi for their chemical armoury, also helping in the survival of host plant and protection and have attracted intense attention due to its biotechnological and pharmaceutical applications. Medicinal plants can be preserved and used for common human ailments. The active ingredients depend upon Medicinal plants have secondary plant metabolites of different composition are grouped as Alkaloids, Glycosides, Terpenoids, Steroids, Saponin and Essential oils etc. Fungal endophytes capable of producing these metabolites can solve the problem of overexploitation of the plants leading to extinction. The plant endophytic fungi are novel mine of natural bioactive compounds with great potentials in agriculture, medicine and food industry. Taking advantage of modern technologies we can better understand and manipulate this important microorganism resource and make it more benefit for the mankind. Thus metabolites from the fungal endophytes of several precious herbs and plants can be developed into medicines in laboratories and can benefit the public. Studies conducted during the last 30 years have shown that presence of endophytic fungi is ubiquitous.[12] Terpenes belong to the biggest class of secondary metabolites and basically consist of five carbon isoprene units which are assembled to each other (many isoprene units) by thousands of ways. Most of the terpenoids with the variation in their structures are biologically active and are used worldwide for the treatment of many diseases. Many terpenoids inhibited different human cancer cells and are used as anticancer drugs such as Taxol and its derivatives.[13] Our findings suggest that Ct from *C.gloeosporiodes* possess significant cytotoxicity, suggesting the possibility of anticancer activity and warrants further scientific investigations. With immense applications, it is worthwhile to study the therapeutic potentials of fungal endophytes (Table 1).

Table 1 Cytotoxicity of Ct in different concentrations in SW620 cells

Concentration of the drug in micrograms	Percentage of inhibition
2	67.6551±2.3123
4	49.7131±2.0446
6	32.0414±2.1406
8	20.74757±2.3099

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Acknowledgments

None.

Conflicts of interest

Authors declare that there are no conflicts of interest.

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