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# *In vitro* cytotoxicity of an endophytic fungus isolated from *Nothapodytes foetida*

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**Abstract -** An attempt has been made to assess *in vitro* cytotoxicity of an endophytic fungus from *Nothapodytes foetida*. Various human cancer cell lines (liver HEP-2, lung A-549, ovary OVAR-5, prostate PC-3, cervix HeLa, colon HCT-15, oral cell line KB, CNS SNB-78, were used. *In vitro* cytotoxicity of camptothecin (CPT) isolated from the fungus was done where OVAR-5 cell line showed maximum inhibition and HEP-2 cell line was least sensitive with this compound. *In vitro* cytotoxicity of fractions/extracts from endophyte was carried out where ethyl acetate fraction showed sufficient growth inhibition against all the cell lines.

**Key words:** *Nothapodytes foetida*; endophytes; cytotoxicity.

## INTRODUCTION

Cancer is the second major cause of deaths after cardiovascular diseases and there is a need to combat such diseases. The search for natural products as potential anticancer agents dates to 1550 B.C. and surveying the period from 1981 to 2002. Newman *et al*., (2003) reported that > 60% of the approved drugs for cancer treatment are natural products or derivatives of natural products. A large number of plants, marine and microbial sources have been tested as leads and many compounds have survived the potential leads. The discovery of camptothecin by Wall and Wani (1966), as an anticancer drug with a unique mode of action that is inhibition of DNA topoisomerase I, added an entirely new dimension to the field of chemotherapy. The compound was originally isolated from the Chinese plant, *Camptotheca acuminate* and in very low yield from Indian plant *Nothapodytes foetida (Mappia foetida)*. Unfortunately there has been over exploitation of these plants species as sources of raw material for production of medicine leading to depletion of a number of plant species. It is necessary to reduce dependency on wild resources and find alternative sources for the production of these important biomolecule. Microbes associated with these plants (endophytes) may be the source of these important bioactive molecules and the way for conserving the important medicinal plants.

Herein we report the production of camptothecin by an endophytic fungus, camptothecin (naturally occurring group of quinoline alkaloid depicting profound cytotoxic activity). We have studied an endophyte for its *in vitro* cytotoxicity studies against human cancer cell lines.

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## MATERIALS AND METHODS

**Isolation of camptothecin (CPT) from endopyhtic fungus.** An endophytic fungus was isolated and purified from stem of *Nothapodytes foetida*. Purified fungus was grown in Sabouraud dextrose broth (dextrose 4% and peptone 1% w/v) at 28 ± 2 °C under shaking with 220 rpm. Dried fungus (700 g) was extracted with a mixture of chloroform and methanol (80:20) in a separating funnel for 3 times. All the extracts were pooled and distilled which yielded 12 g of the residue. The residue obtained from extraction of mycelia (12 g) was subject to column chromatography over silica gel column (600 g, 60-120 mesh) eluted with hexane followed by benzene-ethyl acetate mixtures of five increasing polarity profiles (95:5, 90:10, 80:20, 50:50, 10:90). The column was further eluted with ethyl acetate-chloroform (90:10, 80:20, 70:30, 60:40, 50:50, 20:80) and chloroform-methanol (90:10, 80:20, 50:50) and finally drained with 100% methanol. Fractions of each distinct polarity were pooled and subjected to TLC and HPLC. Positive fractions showing bluish fluorescent spot under UV ($\lambda_{max}$ = 256 nm) with the same retardation factor ($R_f$) as that of standard CPT were pooled and concentrated to give 120 mg of material with some impurities. This mixture was rechromatographed on silica gel (20 g, 60-120 mesh). Elution was started with chloroform, gradually increasing the percentage of methanol in chloroform. The fractions eluted in 1-5% methanol in chloroform showed same $R_f$ as that of standard CPT. These fractions were pooled.

**Growth media for cancer cell lines.**
*Incomplete growth medium.* RPMI-1640 medium (Sigma Chemicals Co., USA) with 2 mM L-glutamine was dissolved in

double distilled water. Streptomycin (100 mg $ml^{-1}$) was also added to medium and pH was adjusted to 7.2. Medium was sterilised by filtering through 0.2 micron filters in laminar flow under sterile conditions. Afterwards medium was stored in refrigerator (2-4 °C).

*Complete growth medium.* Before use, the incomplete growth medium was supplemented with 10% foetal calf serum and penicillin (100 U/ml) to make it complete growth medium.

***In vitro* cytotoxicity of fungal CPT.**

*Cell culture methods*. Tissue culture complete growth medium was used for the growth of human cancer cell lines (liver Hep-2, lung A-549, ovary OVAR 5, prostate PC-3, cervix HeLa, colon HCT-1) at 37 °C in an atmosphere of 5% $CO_2$ and then in 90% relative humidity carbon dioxide incubator. The fresh medium was changed from time to time as per requirement. The fresh medium was brought to 37 °C in a water bath. The medium of the flask was taken out and discarded and fresh medium was placed. The volume of the medium used was dependent upon the capacity of the flask as per manufacturer's instructions.

*Subculture of the cell line.* The medium of the flask having subconfluent growth was changed one day in advance for subculturing. The entire medium was taken out from the flask and discarded. Cells were washed with Phosphate buffered saline (PBS). Trypsin-EDTA in PBS (0.5 ml, pre warmed at 37 °C) was added to make a thin layer on the monolayer of the cells. The flask was incubated for approximately 5 min at 37 °C and observed under microscope. Complete growth medium (1.0 ml, pre warmed at 37 °C) was added, if cells were found to be detached. An aliquot was taken out and the cells were counted and checked for viability with trypan blue. Cell stock with more than 98% cell viability was accepted for determination of *in vitro* cytotoxicity. The cell density was adjusted to 1 x $10^6$ cells/ml by the addition of complete growth medium and was further incubated in $CO_2$ incubator to continue the culture.

*Preparation of test material.* A stock solution of 20 mg of the test material (i.e fungal CPT as described above) in 1 ml of dimethyl sulphoxide (DMSO) was prepared.

Working test solutions were obtained from stock solutions serially diluted with complete growth medium containing 50 µg $ml^{-1}$ of gentamycin to control the microbial contamination.

*Cytotoxicity assay. In vitro* cytotoxicity against human cancer cell lines was determined using 96-well tissue culture plates as described by Monk *et al*., 1991. The cell suspension of required cell density was prepared in complete growth medium with gentamycin for determination of cytotoxicity. The aliquots of 100 µl of cell suspension were added to each well on a 96-well tissue culture plate. The blank wells contained complete medium in place of cell suspension. The cells were incubated at 37 °C for 24 h.

Test material (100 µl in each well) of desired concentrations was added after 24 h to the wells containing cell suspension and blank wells. Simultaneously, control experiments with and without suitably diluted DMSO in place of test material and positive controls containing known anticancer agents like paclitaxel, mitomycin C and flourocil were also carried out. All the experiments were carried out in triplicate.

The cells were allowed to grow in presence of the test material by further incubating the plates for 48 h. At the end of the incubation period the cell growth was stopped by gently layering trichloroacetic acid (50% TCA, 50 µl/well) on top of the medium in all the wells. The plates were incubated at 4 °C for 1 h to fix the cells attached to the bottom of the wells. Supernatant of all the wells was gently pipetted out and discarded. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins, etc., and air dried.

*Sulphorhodamine B (SRB) assay.* The SRB assay was performed to assess cell growth (Skehan *et al.*, 1999), SRB dye (100 µl/well) was added to each well and plates were allowed to stand at room temperature for 30 min then the plates were washed four times with 1% acetic acid. The plates were dried and Tris buffer (100 µl/well) was added to each well to solubilise the dye. The plates were shaken gently for 10 min on a shaker and the optical density was recorded on ELISA reader of Robotic liquid handling system at 540 nm.

*Calculations.* The cell growth was determined by subtracting mean OD value of representative blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated considering the growth in absence of any test material as 100% and in turn percent growth inhibition in presence of test material was calculated.

***In vitro* cytotoxicity of organic extracts.** The chloroform:methanol extract of mycelia (6 g) was prepared as previously described. It was subjected to column chromatography (using 500 g silica gel, 60-120 mesh size) and the graded elution of column was carried out with benzene, ethyl acetate, and methanol. The fractions which showed identical behaviour on TLC were pooled and designated as fractions, ZPF-1 (benzene fraction), ZPF-2 (ethyl acetate fraction), and ZPF-3 (methanol fraction). All the extracts/fractions were studied for their *in vitro* cytotoxicity against human cancer cell lines as described. *In vitro* cytotoxicity of fractions/extracts was determined against six human cancer cell lines: liver (Hep-2), colon (HCT-15), CNS (SNB-78), prostate (DU-145), oral (KB), and lung tissues (A-549) at two concentrations, Flurouracil, Mitomycin C and Paclitaxel were used as standards (Sigma Chemicals Co., USA)

**Endophyte identification.** The fungus, isolated from the inner bark of *Nothapodytes foetida*, was identified by 28S ribosomal gene sequencing. After 48 h of growth in Sabouraud dextrose broth (with constant shaking) at 28 °C, mycelial biomass could be collected in gram quantities.

*DNA extraction.* Total genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method (Rehman *et al*., 2008). Briefly the endophyte was grown in 100 ml Sabouraud dextrose broth at 28 °C with constant shaking for 5 days. Hundred milligrams of mycelial biomass was taken, washed twice with sterile Tris-EDTA buffer, and 6 ml of CTAB extraction buffer and 60 µl of β-mercaptoethanol were added. Afterwards, the mixture was incubated at 65 °C for 45 min, and cooled down to room temperature. This was followed by extraction with equal volume of chloroform and centrifuging at 10000 *x g* for 10 min. Subsequently, equal volume of isopropanol was added to the supernatant and mixed gently. The obtained DNA pellet was washed with icy 70% (v/v) ethanol, vacuum dried and dissolved in 100 µl of TE (pH 8.0).

*Small subunit gene sequencing and analysis.* The endophytic fungus was characterized on the basis of ribosomal gene analysis. The small subunit ribosomal gene was amplified using the D2

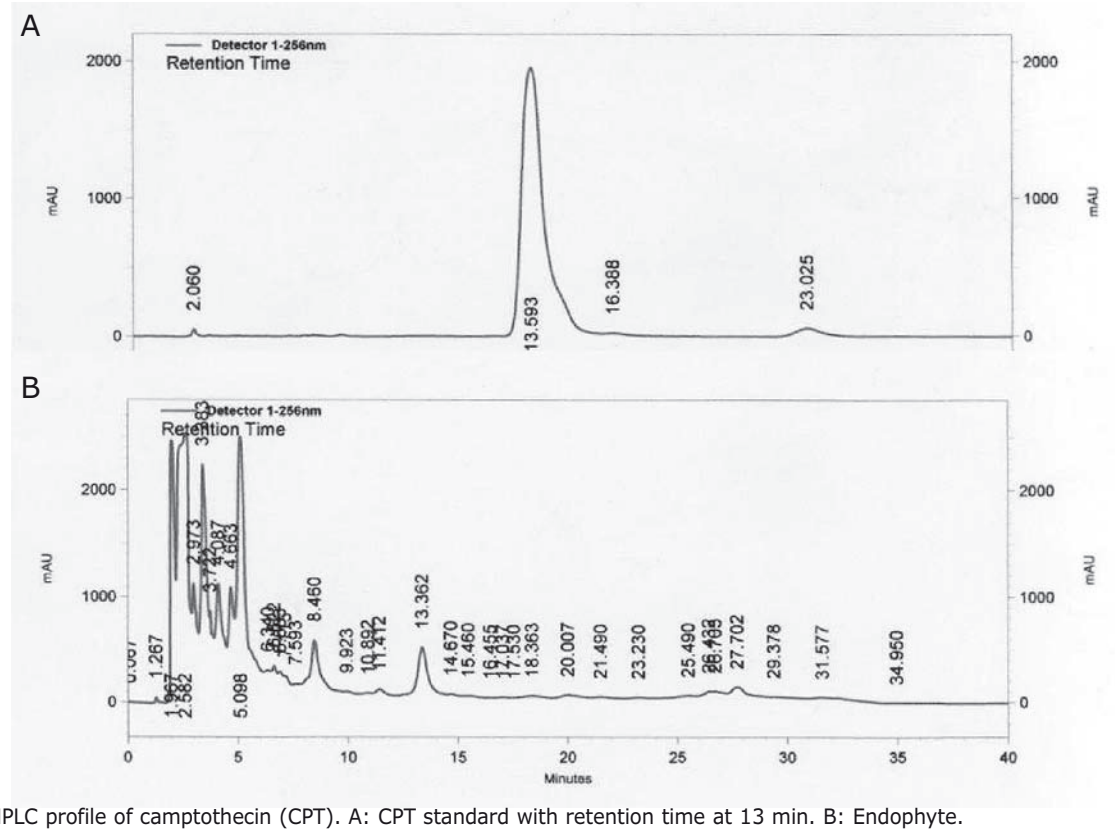


FIG. 1 - HPLC profile of camptothecin (CPT). A: CPT standard with retention time at 13 min. B: Endophyte.

LSU Microseq ki (ABI, USA). The amplified products were purified using Microcon columns (Millipore, USA), and sequenced using ABI Prism310 genetic analyzer (ABI) as per the manufacturer's instructions. The DNA sequence 280 bases (GenBank Acc. No. EU284592) was analyzed for homology studies by BLASTN program (Altschul *et al*., 1997). The ribosomal gene database (http//ncbi.nim.nih.gov) was accessed and sequence alignment was used as an underlying basis to identify the fungus.

## RESULTS

### Isolation of CPT from endopyhtic fungus

Thin layer chromatographic based analysis was performed, and the spots identified at $R_f$ = 0.23 was observed under UV, both sample and authentic camptothecin were florescent at 256 nm. HPLC analysis (Fig. 1) resulted in authentication of camptothecin.

### *In vitro* cytotoxicity studies of fungal CPT

The isolated CPT from the fungus was subjected to *in vitro* cytotoxicity against six human cancer cell lines obtained from four tissues at different concentrations. A concentration dependant growth inhibition was observed against all the cell lines. The degree of growth inhibition was cell specific. The OVAR-5 cell line of the ovary showed maximum inhibition of 90% where as Hep-2 (liver) cell line was least sensitive showing only 25% inhibition, detailed results are given in Table 1.

### *In vitro* cytotoxic studies of fungal organic extracts

The ethyl acetate fraction with maximum inhibition of 90% was observed against Hep-2 cell line. This also showed sufficient growth inhibition against all the cell lines. The fractions obtained with methanol and benzene did not show any appreciable activity at both concentrations (Table 2).

### Endophyte identification

Figure 2 shows the distance tree constructed on the basis of homology of 28S ribosomal gene sequence of endophytic strain with close members in GenBank. The endophyte could not be assigned to a genus and a species but it showed highest sequence similarity of 95% with *Nodulisporium* sp. followed *by Hypoxylon fragiforme* and *Daldinia concentrica*, both sharing a sequence similarity of 93% with the endophyte.

TABLE 1 - *In vitro* cytotoxicity of fungal camptothecin (CPT)

| Fungal CPT concentration (M) | Cytotoxicity (%) | | | | | |
|---|---|---|---|---|---|---|
| | Liver Hep-2 | Lung A-549 | Ovary OVAR-5 | Prostate PC-3 | Cervix HeLa | Colon HCT-15 |
| $1 \times 10^{-8}$ | 19 | 68 | 55 | 1 | 19 | 0 |
| $1 \times 10^{-7}$ | 20 | 71 | 69 | 28 | 30 | 18 |
| $1 \times 10^{-6}$ | 23 | 77 | 72 | 27 | 33 | 20 |
| $1 \times 10^{-5}$ | 25 | 87 | 90 | 33 | 41 | 28 |

The *in vitro* cytotoxicity was determined as per method described in methodology. The values reported are the mean of two experiments.

TABLE 2 - *In vitro* cytotoxicity of organic extract/fractions of fungal mycelia against human cancer cell lines compared with positive controls containing known anticancer agents (flourocil, mitomycin C and paclitaxel)

| Fractions* | Concentration (µg/ml) | Cytotoxicity (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | CNS SNB-78 | Liver Hep-2 | Lung A-549 | Oral KB | Prostate DU-145 | Colon HCT-15 |
| ZPF-3 | 10 | 27 | 19 | 68 | 23 | 10 | 0 |
| | 30 | 23 | 33 | 45 | 35 | 14 | 7 |
| ZPF-2 | 10 | 70 | 90 | 71 | 50 | 62 | 40 |
| | 30 | 54 | 91 | 69 | 48 | 59 | 35 |
| ZPF-1 | 10 | 17 | 23 | 65 | 13 | 15 | 20 |
| | 30 | 12 | 11 | 19 | 20 | 19 | 12 |
| Anticancer agents | Concentration (M) | | | | | | |
| Flourocil | $2 \times 10^{-5}$ | | | | | | 30 |
| Mitomycin C | $1 \times 10^{-5}$ | | 65 | | 80 | | |
| Paclitaxel | $1 \times 10^{-5}$ | 47 | | 66 | | 85 | |

* ZPF-3: methanol fraction, ZPF-2: ethyl acetate fraction, ZPF-1: benzene fraction.
The *in vitro* cytotoxicity was determined as per method described in methodology. The values reported are the mean of two experiments.

## DISCUSSION

In this study, the biological importance of the fungal originated camptothecin possessing anticancer activity was established. A concentration dependant growth inhibition was observed against all the cell lines. CPT and other solvent fractions from the endophyte which showed appreciable activity against the cell lines could be considered for cancer therapy. Major concern to the medical community is the latent toxicity of any prospective drug to the higher organisms such as human tissues. It would appear that since the plant is also a eukaryotic system in which the endophyte exists, the metabolites made by the endophyte may have reduced cell toxicity; otherwise, death of the host tissue may occur. Thus, the plant itself has naturally served a selection system for microbes having bioactive molecules with reduced toxicity towards higher organism.

The present study is based on the premise that screening of beneficial endophytic fungus possess enhanced anticancer capabilities. It is only the indication for alternate source of camptothecin supply for the production of potent anticancer analogue. But detailed study would be required to further develop the isolated fungal strain as a potential organism for the economic production of camptothecin. Thus, the study of such indigenously effective fungal flora with broad-spectrum biological activities are perceived as a viable and cost effective approach for developing a genetic resource.

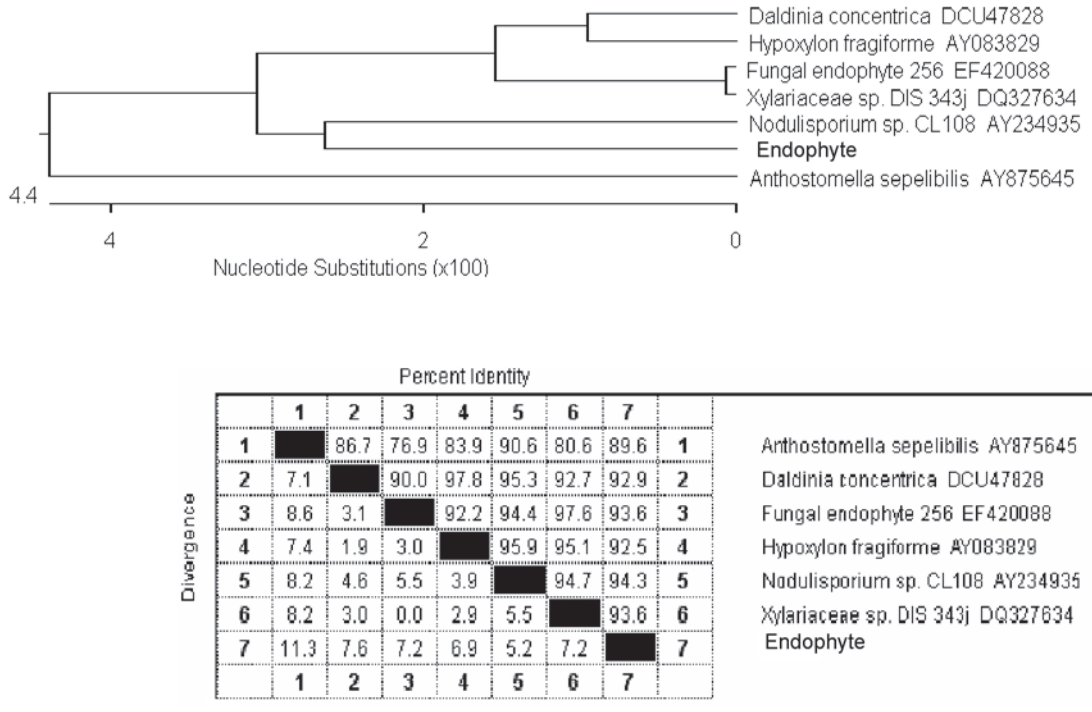


FIG. 2 - Phylogenetic position of the endophyte strain.

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