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# *IN VITRO* ANTICANCER ACTIVITY OF ENDOPHYTIC FUNGAL EXTRACTS FROM *CLADOSPORIUM* FAMILY ISOLATED FROM *CYATHOCLINE PURPUREA* (D. DON.) O. KTZE

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## ABSTRACT

*Cyathocline purpurea* (D. Don.) O. Ktze. (Asteraceae) is a well documented anticancer plant, demonstrating activity against breast cancer cell line- MCF-7. In our present investigation, comparison of cytotoxic potential of 13 extracts from 5 endophytic *Cladosporium* species namely *Cladosporium sphaerospermum* Penz, *Cladosporium* aff. *cladosporioides* (Fresen.) de Vries, *Cladosporium cladosporioides* (Fresen.) de Vries and *Cladosporium oxysporum* Berk. and M. A. Curtis. isolated from leaves of *C. purpurea* were studied. Axenic cultures of *Cladosporium* species were isolated from *C. purpurea* leaves following surface sterilization of leaves. Radial growth in agar plate was compared using different media viz. SDA, SDMA, CDA, YESA and PDA and accordingly SDA/SDB was selected. Culture were grown in SDB media and extracts were prepared using different solvents viz. EA, CHL, HD and HA extract to ascertain cytotoxicity against MCF-7 breast cancer cell line. Most of the *Cladosporium* fungal extracts demonstrated good cytotoxicity against MCF-7 estrogen positive breast cancer cell line. Interestingly, *Cladosporium cladosporioides* (Fresen.) de Vries which shares affinity with *Cladosporium* aff. *cladosporioides* (Fresen.) de Vries at the species level, fared better in MTT cytotoxic profile with respect to MCF-7 breast cancer cell line. Further analysis of these 2 strains may shed light on correlation of their genetic profile with respect to their cytotoxic potential. Our results indicated that use of *Cladopsporium* extract can be a good secondary source for possible antineoplastic agent. However, further work is required for screening their potency against various cell lines, both in cancer and in normal.

**KEYWORDS:** *Cyathocline purpurea*; MTT; *Cladosporium*; cytotoxicity; MCF-7; media optimization.

## INTRODUCTION

The term endophyte coined by German scientist Heinrich Anton De Bary[1] defines them as a group of microbes, residing in living tissues, without causing any kind of physical disease manifestation in host.[2] Occasionally, endophytes have been documented as a prolific producer of an array of compounds, both known and unknown, having application in the field of medicine, agriculture and industries. They have the potential to be explored in the form of crude extracts and sources of secondary metabolites as cytotoxic agents.[3]

*Cyathocline purpurea* (D. Don.) O. Ktze. (Asteraceae), is a well-known rare herb, mostly used indigenously as germicide and appetizer. Antimicrobial, antiprotozoal and pharmacological activities have been reported for *C. purpurea*.[4] *In vivo* peripheral analgesic and anti-inflammatory effect in rodents and *In vitro* antioxidant activity was also demonstrated in *C. purpurea*.[5] Fractions of methanolic extract from *C. purpurea* yielded isoivangustin, a sesquiterpene lactone which exhibited anti-inflammatory activity in wistar rats.[6]

Traditionally, *C. purpurea* has been documented for having anticancer properties. Santamarine, 9beta-acetoxycostunolide and 9beta-acetoxyparthenolide, isolated from *C. purpurea* exhibit significant anticancer activities *in vitro* with IC(50) within the range of 0.16-

1.3 µg/mL against MCF-7, L1210 murine leukaemia, CCRF-CEM human leukaemia, KB human nasopharyngeal carcinoma, LS174T human colon adenocarcinoma.[7]

Breast cancer is one the malignant cancers affecting women worldwide and 2nd leading cause of death, in cancer related deaths. There is an increase in number of cases, upto 3.1% annually.[8]

The availability of taxane drugs viz. Tamoxifen has received positive response for breast cancer treatment, specifically for estrogen positive breast cancer. But due to development of multi drug resistance among patients, it is the need of the hour to find alternate drugs for effective treatment for cancers.[9] In the process it is noted that, atleast 60% of the drugs and compounds used in anticancer treatment are obtained from plant or microbial source.[10]

The increased use of anticancer metabolites isolated from plants, resulting in large scale destruction leading to environmental and conservation issues. Therefore, fungal components of endophyte origin offer a relatively cheaper and environmental friendly mode of obtaining useful compounds. As there are no reports available on screening endophytic fungal extracts from various *cladosporium* species against MCF-7 estrogen positive breast cancer cell lines, our present study forms its first report, which will be of great implication in developing downstream applications.

## MATERIALS AND METHODS

### Sterilization procedure

Healthy leaves were collected from *C.purpurea* from Kangrali area in Belagavi and processed immediately for isolation.

The standard sterilization procedures were followed with minor modifications.[11] The collected leaves were surface sterilized with 0.01% tween 20 solution, dipped in 70% ethanol, followed by dipping in 90% ethanol. This was repeated thrice. Finally the leaf materials are rinsed with DDW (double distilled water) and dried in LAF (laminar air flow hood). To ascertain the effectiveness of sterilization procedure and eliminating epiphytic fungi, if any, leaf imprinting on PDA (potato dextrose agar) was done.[12]

### Isolation and identification

1gm leaves were macerated in 10ml of DDW in sterile mortar pestle. 1ml of each of which was transferred to PDA, SDA, YESA, CDA and SDMA with streptomycin incorporated in it (20mg/1000ml media). This was to obtain all the possible diversity among fungi and for eliminating bacterial isolates. These plates were incubated at 28˚C for 15 days. Each fungal isolates were further sub-cultured in PDA, SDA, YESA, CDA and SDMA separately without antibiotic to assess its media suitability. The entire procedure was repeated thrice in aseptic conditions. SDA/ SDB was selected as the media of choice based on the assessment. Later, the axenic cultures obtained, were maintained in FA slants, sealed and stored at 4˚C.

For tentative identification, cultures were identified using lactophenol cotton blue stain using light microscope, Olympus, USA.The axenic culture obtained were sent for confirming the morphological identity to National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune. The strains were identified as *Cladosporium sphaerospermum* Penz, *Cladosporium* aff. *cladosporioides* (Fresen.) de Vries, *Cladosporium cladosporioides* (Fresen.) de Vries and *Cladosporium oxysporum* Berk. and M. A. Curtis. respectively.

### Preparation of extract

Standard procedure was followed with slight modification. Agar blocks (50mm) were used as inoculum in SDB broth from SDA agar and incubated for 28˚C for total period of 30 days in 12 hours light/ dark. After incubation period, mycelia was washed and soaked in 70% ethanol, followed by maceration to obtain HA extract. The filtrate broth was used to prepare EA (ethyl acetate), CHL(chloroform) and HD (hexane:dichloromethane, 1:1) extract. 50/1000ml of solvent was added to filtrate broth and left submerged for 3 days and later extracted with 50ml of solvent. Extraction was done three times for each solvent. The extracts of each solvent was pooled and dried with rotatory evaporator (Heidolph- Heivep Advantage, Germany) under vacuum at 40˚C until dry extract was obtained. The dried extract was weighed and stored in glass vial at -20˚C till further use.[13]

### Cytotoxicity activity using MTT assay

Standard MTT assay was followed with slight modification to analyse cytotoxicity activity quantitatively.[14,15] Briefly, MCF-7 breast cancer cell lines were seeded in 96 well plate containing media DMEM (dulbecco's modified eagle medium) coupled with 10% heat inactivated FCS (fetal calf serum), along with 5% of mixture of Gentamicin (10µg), Penicillin (100 Units/ ml) and Streptomycin (100µg/ml) in presence of 5% $CO_2$ at 37˚C for 48 to 72 hours. The cells were seeded in 96 well plate at a concentration of 2.0 X $10^4$ in 100µl per well of medium. Different concentrations of drug ranging from 10 to 160µg/ml were added to seeded wells and were incubated at 37°C in 5% $CO_2$ with humidity more than 94% for a period of 24 hours. PBS (phosphate buffered saline) containing 20µl of MTT (stock 5mg/ml) was later added and incubated for 4 hours. The supernatant was aspirated and about 100µl of DMSO (dimethyl sulfoxide) was added for solubilisation of formazan crystals. The absorbance of treated versus untreated cells gave an estimate of viability of cells. Equivalent concentration of DMSO in treated cells were used as negative control. At 492nm, OD (optical density) was calculated, using ELISA reader (Thermo scientific, USA) and $IC_{50}$ (inhibitory

concentration by 50%) value was deducted, using the following formula:[16]

$$\text{Cell viability}(\%) = \frac{\text{Mean OD of the test compound} \times 100}{\text{Mean OD at control}}$$

$IC_{50}$ value was derived by plotting % cytotoxicity/ growth inhibition against drug concentration (in µg/ml) using MS-excel worksheet.[17]

Percent cytotoxicity was derived using formula: [1-(test/control)] x 100

## RESULT AND DISCUSSION

A total of four endophytic *Cladosporium* species were isolated from *Cyathocline purpurea* (D. Don.) O. Ktze (Asteraceae). Mycelia was inoculated and were grown in different media (PDA, SDA, YESA, SDMA and CDA) to assess their colony morphology and media suitability via radial growth assessment in agar plate. There was no observable change noticed in morphology due to change in media on agar plate. Accordingly SDA was selected as media of choice and SDB was used for further mass culture of these selected fungal strains table as presented in Table.1.

**Table 1: Media suitability based on radial growth parameter in agar plate.**

| Endophytic fungi | Colony morphology | Radial growth (mm) | | | | |
|---|---|---|---|---|---|---|
| | | SDA | PDA | SDMA | CDA | YESA |
| *Cladosporium cladosporioides* (Fresn.) de. Vries | Dark grayish green with black reverse | 40 | 34 | 32 | 36 | 37 |
| *Cladosporium* aff. *cladosporioides* (Fresn.) de. Vries | Dark grayish green with black reverse | 45 | 36 | 35 | 38 | 43 |
| *Cladosporium sphaerospermum* Penz. | Dark grayish green velvety surface with black reverse | 80 | 65 | 75 | 36 | 58 |
| *Cladosporium oxysporum* Berk and M.A. Curtis | Green velvety surface with black reverse | 43 | 35 | 34 | 39 | 30 |

HD, EA, CHL and HA extracts were prepared from all the four axenic cultures of endophytic fungal isolates. Among these, thirteen extracts exhibited cytotoxicity in MTT assay as shown in Figure 1- 3. Doxorubicin was administered to MCF-7 cell lines, at different concentrations (1 to 6 µg/mL), induced significant inhibition as compared to control. Dox was highly cytotoxic, and the reduction in cell viability was observed at 3.7 µg/mL in Dox treated MCF-7 cell lines.

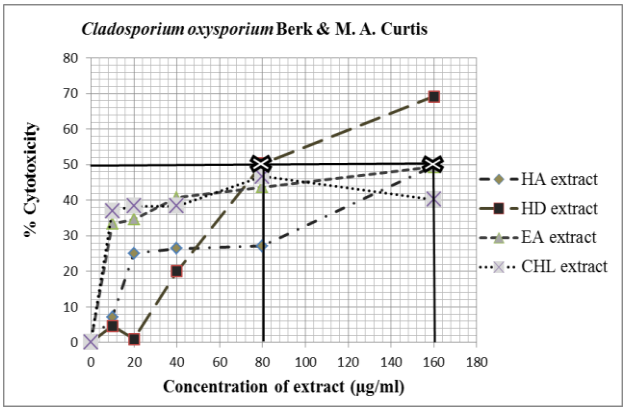


**Figure 1: *Cladosporium oxysporum* Berk and M. A. Curtis HA, HD, EA and CHL extracts exhibited cytotoxicity at 160 µg/ml, 80 µg/ml, 160 µg/ml and 80 µg/ml respectively.**

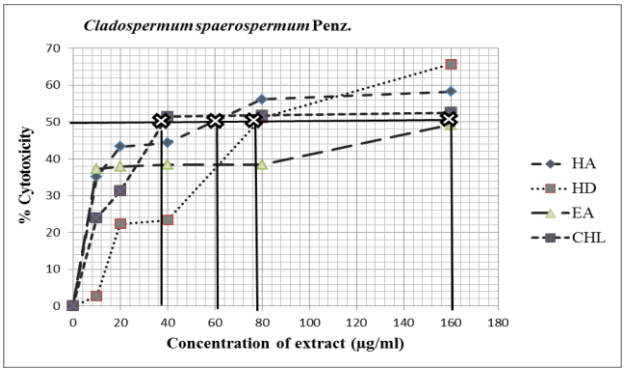


**Figure 2: *Cladosporium spaerospermum* Penz. HA, HD, EA and CHL extracts exhibited cytotoxicity at 60 µg/ml, 78 µg/ml, 160 µg/ml and 36 µg/ml respectively.**

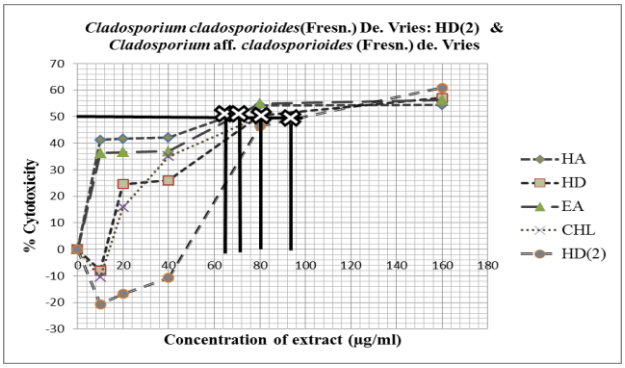


**Figure 3: Extracts of *Cladosporium* aff. *cladosporioides* (Fresn.) de Vries: HD(2) exhibited 96 µg/ml while HA, HD, EA and CHL extracts of *Cladosporium cladosporioides* (Fresn.) de Vries demonstrated toxicity at 64 µg/ml, 80 µg/ml, 68 µg/ml and 80 µg/ml respectively.**

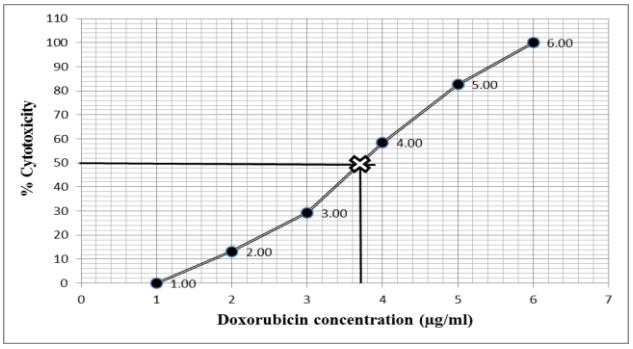


**Figure 4: Doxorubin (standard) used, exhibited cytotoxicity of 3.7 µg/ml against *Cladosporium* endophytic fungal extracts.**

Earlier reports of *Cladosporium* species demonstrated antioxidant property via DPPH test.[18] Bio activity guided fractionation yielded several compounds including 5′-hydroxyasperentin and cladosporin-8-methyl ether which demonstrated antifungal properties.[19] *Cladosporium uredinicola*, isolated from *Psidium guajava* fruit also led to production of antimicrobial depside.[20] However, the present study forms the first report of *Cladosporiun* species being isolated from *C. purpurea* and also the first report for demonstrating the anticancer activities of their extracts in MCF-7 cell line.

## CONCLUSION

Our results indicate anticancer potential of crude extract from endophytic *Cladosporium* fungal species. Further work is essential to validate its anti-cancer potential using other types of breast cancer cell lines and spectroscopic studies. It is also possible that the extracts which did not exhibit activity in MTT assay for MCF-7 cell line may show positive results in other breast cancer cell lines. It is noteworthy that *Cladosporium cladosporioides* (Fresen.) de Vries, which is similar to *Cladosporium* aff. *Cladosporioides* (Fresen.) de Vries at the species level, performed better in MTT cytotoxic profile in the present experimental setup.. Further molecular analysis and genetic profiling of these two strains may help to analyse the extent of similarities and differences among them and correlating their cytotoxic potential.

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