Int J Pharm Bio Sci 2012 Oct; 3(4): (B) 533 - 540

**Research Article** **Biotechnology**

**International Journal of Pharma and Bio Sciences** **ISSN 0975-6299**

# PHYTOCHEMICAL ANALYSIS, ANTIMICROBIAL AND ANTITUMOUR SCREENING OF ENDOPHYTES OF TINOSPORA CORDIFOLIA

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## ABSTRACT

*Tinospora cordifolia*, commonly known as 'Guduchi' in India is a medicinal plant which is used to treat various diseases. Phytochemical analysis of plant revealed the presence of alkaloids, flavonoids, steroids, saponins, total phenols, cardiac glycosides and reducing sugars. The total phenol content was 0.39 mg GEA/mg, 0.25 mg GEA /mg for leaf and stem respectively. These findings could be useful for both pharmaceutical companies and research institutes in the development of new drugs.

Out of nine endophytic fungal isolates, eight belonged to *Penicillium* sp. and one remained unidentified. Fungal extracts were tested for antimicrobial and antitumor activity. Result showed that endophytes had good antibacterial activity compared to antifungal activity. Antitumor activity assayed by SRB assay method using MCF and SiHA cell lines was negative for endophytes selected. Since endophytes are potent source of the secondary metabolites and also have antibacterial property they can be used for pharmaceutical applications.

**KEYWORDS: Endophytes, antitumour, total phenol content, antimicrobial, *Tinospora***

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# INTRODUCTION

Medicinal plants are natural source of drug molecules since they contain active compounds, properties of which are beneficial to humans. Since ancient times, medicinal plants have attracted researchers for identification of active biomolecules having important biological activities. The medicinal value of these plants is due to bioactive constituents of plants which include alkaloids, tannin, flavonoids and phenolic compounds. These bioactive chemicals produce a definite physiological action on the human body [1].

Considering the importance of medicinal plants in the field of medicine, an investigation was undertaken for screening of *Tinospora cordifolia*. This medicinal plant belongs to family Menispermaceae. The plant contains various secondary metabolites like tinosporine, tinosporide and β-sitosterol. Phenylpropanoids, norditerpene furan glycosides, diterpene furon glycosides and plytoecdysones are present in methanolic extract of plant. The extracts of plant have demonstrated immunological activity. The stem is used in dyspepsia, fevers and urinary diseases. The bitter principle of plant has various properties like antiperiodic, anti-inflammatory antispasmodic and antipyretic properties. Plant Guduchi has potential active ingredient to treat throat cancer in humans.

The most commonly used definition of "endophytes" is "All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host [2]. These endophytes, residing within plant tissue, are exploited in the field of medicine, agriculture and pharmaceutical industry since they are the source of novel natural products. Fungal endophytes attract researchers because of their chemical diversity [3].

Endophytic fungi are the underexplored group of microorganisms which are the promising source of novel products like antibiotics, antibacterial agents, antifungal agents, antitumor agents etc. The potential prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great.

Considering the vast potentiality of medicinal plant and endophytes as a source of drug for therapeutic application, an investigation was undertaken to screen various phytochemicals from the plant *Tinospora cordifolia* and to study antimicrobial and antitumor properties of endophytes.

# MATERIALS AND METHODS

### *Plant material*

Medicinal plant *Tinospora cordifolia* collected from University of Agricultural Science, Dharwad and forest colony, Hubli were identified and obtained. Healthy and mature plants were carefully chosen for sampling. Samples were collected randomly and brought to the laboratory in sterile bags. Leaf and stem from these plants were gathered and stored aseptically.

### *Preparation of plant extract*

The samples were cleaned thoroughly and sun dried for 8-10 days. The dried samples were macerated with methanol and allowed to stand for 48 hours (10 gm in 100 ml methanol) and filtered. The filtrate was then evaporated in hot air oven and extracted using methanol. The extracts were stored in refrigerator at 5°C and used for further analysis.

### *Phytochemical Analysis*

The extract of plants were analyzed for presence of alkaloid, saponin, reducing sugars, steroids, flavonoid, cardiac glycoside and total phenol content according to standard methods [1,4,5,6.]

### *Total phenol content*

Total phenols content was determined by the Folin–Ciocalteu method [7] with slight modification. The 150 µL of plant extract, 2400

µL of nanopure water, and 150 µL of 0.25 N Folin–Ciocalteu reagent were combined in a test tube, mixed well using a vortex. The mixture was allowed to react for 3 minutes, then 300 µL of 1N $Na_2CO_3$ solution was added and mixed well. The solution was incubated at room temperature in the dark for 2 hours. The absorbance was measured at 725 nm using a spectrophotometer. Gallic acid (0.02–0.1 mg/mL) was used as standard.

***Isolation of endophytic fungi and identification***

Collected plant material was washed and cleaned with detergent. Surface was sterilized by treating it with 70% ethyl alcohol for 2 minutes, washed with distilled water and dipped in 10% sodium hypochlorite for 1-2 minutes. It was again washed with distilled water and treated with 0.1 mg/ml streptomycin for 1 minute. These sterilized samples were placed in a petri plate and samples were cut into small pieces of dimensions 1 cm by tissue tear method with the help of sterile blade and forceps. Cut pieces of sample were placed on Petri plates containing potato dextrose agar (PDA) medium, pre-incubated at 25°C for 5 days. Inoculated PDA plates were then incubated at 25-27 °C for 8-10 days. Fungal growth was observed and individual hyphal tips of fungi were re-inoculated on fresh PDA medium, and incubated at 25-27 °C for at least 12-14days.

Fungal identification methods were based on the morphology of the fungal culture and characteristics of spores with the help of microscopic studies[8].

***Preparation of extract***

Isolated endophytic fungi were cultured on potato dextrose broth and after required incubation period, the fungal crude extract was filtered through sterile cheese cloth to remove the mycelia mats**.** The fungal metabolites were extracted by solvent extraction procedure where ethyl acetate was used as an organic solvent. Equal volumes of the filtrate and ethyl acetate were taken in a separating funnel and shaken vigorously for 10 minutes. The solution was then allowed to stand, where the cell mass got separated and the solvent so obtained was collected. Ethyl acetate was evaporated and the resultant compound was dried in vacuum evaporator to yield the crude extracts. The crude extracts were then dissolved in Dimethyl sulphoxide (DMSO) and used for further studies [9].

***Antibacterial activity by agar disc diffusion method***

Four bacterial strains *Staphylococcus aureus* NCIM 2071*, Pseudomonas aeruginosa* NCIM 2053*, Escherichia coli* NCIM 2065and *Bacillus subtilis* NCIM 2724 were used to determine the antibacterial activity of the cultured broth. Prior to testing, these organisms were cultured in nutrient broth individually. Known volume of this inoculated broth was spread uniformly over Mueller Hinton media plates. Whattman paper discs (4 mm) were saturated with fungal extract and DMSO, allowed to dry and was introduced on the upper layer of seeded agar plates. DMSO and Streptomycin (3 mg/ml) were used as negative and positive control respectively. Plates were incubated at 35±1° C and observed for zone of inhibition[10].

***Antifungal activity***

The test organisms *Aspergillus niger* NCIM 1196 and *Candida albicans* NCIM 3471 were inoculated onto sabouraud dextrose agar. Sterile cork borer was used to bore 5 holes on the agar plates, after which 60 µl of broth were introduced aseptically into the wells of the agar plates seeded with the test organisms. Nystatin (2.5 mg/ml) was used as the positive control. The plates were incubated at 35±1 ° C and observed for zone of inhibition [11].

***Antitumor activity by sulphoramide (SRB) Assay:***

Potential antitumor activity and cytotoxicity of crude extract dissolved in DMSO was tested using SRB technique. Tumor cells were plated in 96 – multi-well plate ($10^4$cells/well) for 24 hrs before treatment with extract to allow

attachment of cell to the wall of the plate. Then, different concentrations of the extract (10, 20, 40, 80 µg/ml) were added to the cell monolayer triplicate wells after prepared for each individual dose. Appropriate positive controls (ADR) were run in each experiment and each experiment was repeated thrice. Monolayer cells were incubated with the compound for 48 hrs at 37 °C and in atmosphere of 5% $CO_2$. After 48 hrs, cells were fixed, washed and stained with Sulfo – Rhodamine –B stain (SRB). Excess of stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. The color intensity was measured in an ELISA reader[12]. Results are given in terms of GI50, TGI and LC50 values.

# RESULTS

***Phytochemical Screening of plant materials***

Phytochemical investigation of the plant indicates the presence of various secondary metabolites as shown in Table 1. *T. cordifolia* tested positive for all tested metabolites (reducing sugar, alkaloids, saponins, cardiac glycosides, steroids and flavonoids) except for tannins. Total phenol content for leaf and stem sample was found to be 0.39 mg GAE/mg and 0.25 mg GAE/mg of leaf and stem respectively.

**Table 1.**
***The phytochemical screening of the crude methanolic extracts of leaves and stem of plants.***

| Plant extracts | Reducing sugar test | Saponin Test | Alkaloids Test | Flavanoid Test | Tannin Test | Cardiac glycoside Test | Steroid Test |
|---|---|---|---|---|---|---|---|
| *T.cordifolia Leaf* | + | + | + | + | - | + | + |
| *T.cordifolia* Stem | + | + | + | + | - | + | + |

***Isolation of Endophytic Fungi***

Leaves and stem of plants were used for isolation of endophytic fungi. Nine fungi were obtained and eight fungi belonged to *Penicillium* sp. as observed by microscopic tests (SRVK 112, 113, 118, 138, 139, 140, 141 and 147). One isolate remained unidentified (SRVK 114). Since entophytes have inherent property of production of secondary metabolites, the isolated fungi were tested for antimicrobial activity and production of bioactive agents.

***Antimicrobial activity***

Three endophytic fungi produced certain pigments on agar plates hence they were selected for further studies. It was observed that endophytic fungi had good antibacterial property against *Bacillus subtilis* for endophytic fungi SRVK 113 (10 mm) and SRVK 141 (7.2 mm). SRVK 147 showed inhibition zone of 10.2 mm against *Staphylococcus aureus*. None of the selected isolates showed positive results for antifungal activity.

**Table 2**

***Table showing inhibition zone (dia: mm), antibacterial activity of isolates against standard organisms.***

| Isolates | *Escherichia coli* | *Staphylococcus aureus* | *Pseudomonas aeruginosa* | *Bacillus subtilis* |
|---|---|---|---|---|
| SRVK 141 | 7 | - | 7 | 7.2 |
| SRVK 113 | - | - | 6.8 | 10 |
| SRVK 147 | - | 10.2 | - | - |

***Antitumour property***

Test cell lines used were SiHA (cervix human origin) and MCF (breast cancer.) All the test samples were negative in the assay system used (Table 3, Graph1, 2). One reason of samples being negative may be that samples were too diluted or solvent solubility problem. Other isolated endophytic fungi can also be tested for antitumor property

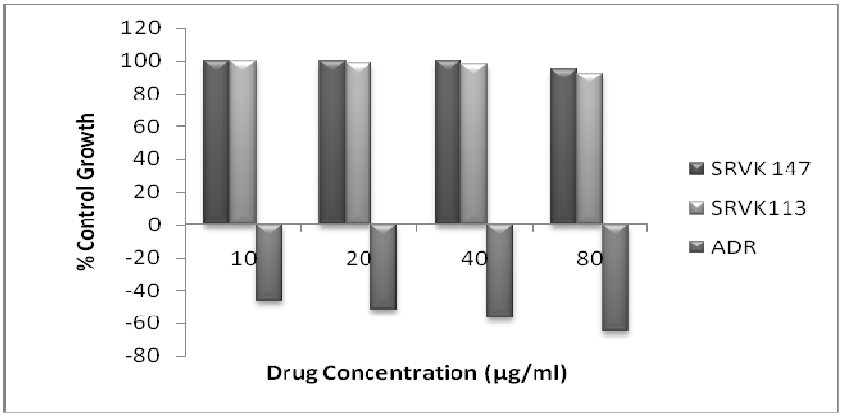
.

**Table 3**

***Table showing the results of Invitro testing of antitumor property using MCFand SiHa cells.***

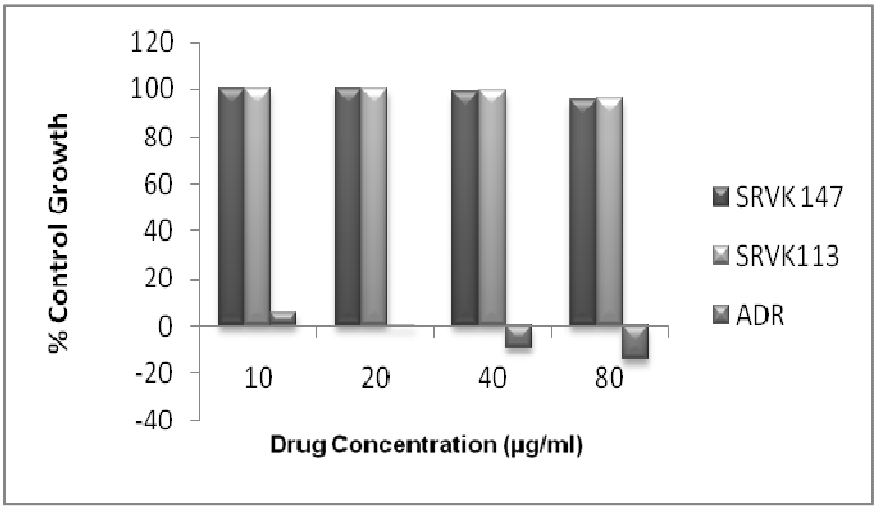
| Sample | Cell Type | LC50 | TGI | GI50 |
|---|---|---|---|---|
| SRVK 141 | MCF | >80 | >80 | >80 |
| SRVK 113 | SiHA | >80 | >80 | >80 |
| | MCF | >80 | >80 | >80 |
| SRVK 147 | SiHA | >80 | >80 | >80 |
| | MCF | >80 | >80 | >80 |
| ADR* | SiHA | 50.42 | 31.10 | <10 |
| | MCF | >80 | 47.82 | <10 |

*ADR=Adriamycin, a positive control, LC50- lethal concentration (the concentration that kills 50% of treated cells). 50% Lethal Concentration; TGI- total growth inhibition (the concentration required to completely halt the growth of treated cells). and GI50- inhibition of cell growth (the concentration needed to reduce the growth of treated cells to half that of untreated cells).




**Graph 1**

***Graph showing the growth curve of human cervix cancer cell line SiHa against extract.***




**Graph 2**

***Graph showing the growth curve of human Breast cancer cell line MCF-7 against extract.***

## DISCUSSION

The results showed that the chemical constituents reported from the plant *Tinospora* belonged to different classes such as alkaloids, flavanoids, glycosides, steroids, terpenoids, phenolics and saponins [13]. The presence of these phytochemicals is an indicator that the plant can be a potential source of precursors in the development of synthetic drugs.

Plant steroids are known to be important for their cardiotonic activities; they possess insecticidal and antimicrobial properties. They are also used in nutrition, herbal medicine and cosmetics; they are routinely used in medicine because of their profound biological activities [5].

It is presumed that the endophytic fungi isolated from medicinal plants have some inherent property from the medicinal plant. Endophytic fungi isolated from various plants are promising source for antitumor and antimicrobial activity [10, 14,3,15]. Endophytic fungi can be used for production of bioactive agents [16,17,18]. Since there are no earlier reports on the endophytes from this plant, hence an attempt was made to isolate and test the isolated fungi for anti- microbial activity and antitumor property.

It was observed from previous reports that the leaf extract showed maximum antibacterial activity compared to other parts[19], methanolic extract of plant sample showed maximum activity [20], and soluble fraction of the methanolic extract of plant showed antibacterial activity[21]. It was also observed that the plant sample has less antifungal activity compared to standard[19]. In the present study, extract from isolates showed good antibacterial activity but no antifungal activity. This is the first report of endophytic fungi from *Tinospora* and its antimicrobial activity.

In the present study of endophytic fungi, none of extracted sample had antitumor property where as it was observed that carbon tetrachloride extract of plant show high levels of antimicrobial activity and cytotoxicity compared to that of chloroform extract or petroleum ether extract [20].

The reasons for observed results for antitumor property may be that the sample was too diluted or solvent solubility problem. Even other assay methods can be followed. Other endophytic fungi may have antitumor property and hence should be tested. It is noteworthy that the extract of endophyte showed antibacterial property.

## CONCLUSION

*Tinospora cordifolia*, an important medicinal plant has shown positive results for the tested phytochemicals except for tannins. Total phenol was also estimated. Most of the isolated endophytic fungi belonged to *Penicillium* sp. Extract from the isolated fungi showed good antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. They may have potent application in biotechnological or pharmaceutical processes.

## ACKNOWLEDGEMENT

The authors thank Karnataka State Council for Science and Technology (KSCST), IISC, Bangalore for partial funding of the project and Advanced Centre for Treatment Research and Education in Cancer, Mumbai for antitumour testing. The authors also acknowledge encouragement from Dr.Ashok Shettar, Principal BVBCET, Hubli.

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