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Research Article

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In vitro Evaluation of the Efficacy of Leaf and its Callus Extracts of *Tinospora cordifolia* (Willd.) Hook.F.Thoms on Pathogenic Fungi

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ABSTRACT

The investigation is aimed to carry out the antifungal activities of ethanol extracts of leaf,stem, leaf derived callus and stem derived callus of Tinospora cordifolia (Willd.) Hook.F.Thoms (Menispermaceae). The leaf and stem segments of Tinospora cordifolia were cultured on MS medium supplemented with auxins and cytokinins alone. In the case of leaf derived callus, maximum biomass was recorded on medium containing 2,4-D, NAA and BAP combination while NAA and BAP combination showed good response for callus induction in stem. The antimicrobial activities of ethanol extracts of leaf, stem, leaf derived callus and stem derived callus were screened against ten fungal species using poisoned food technique. All the tested extracts were bioactive with inhibition in germination of fungal spores.

Keywords: *Tinospora cordifolia, callus culture, antifungal activity,MS medium, poisoned food technique.*

INTRODUCTION

During the past two decades, life-threatening fungal infections are frequently emerging through opportunistic infections with advent of cancer chemotherapy, organ transplantation or HIV[12]. Most of systemic fungal infections are mainly caused by opportunistic pathogens such as *Candida* and *Aspergillus* species[11]. Although many researchers have carried out intensive studies in attempts to develop new antifungal agents and some drugs are under clinical trials. Amphotericin B and the Azole compounds remains mainstay of fungal systemic infection therapy. However, the adverse effects of Polyenes and emergence of *Candida* strains resistant to commercial Azole compounds make the treatment of patients with deeply invasive mycoses difficult. Therefore, demand for the development of new antifungal agents which have low side effects and broad spectrum activity against various fungi is greater than ever before.

Plants have been an important source of medicine for thousands of years. Even today, the World Health Organization (WHO) estimates that up to 80 percent of people still rely mainly on traditional remedies for their medicines[2]. Plants are also the source of many modern medicines. A variety of compounds with antifungal activity are present in plants[1]. In recent past there have been several reports which reveals the presence of fungistatic substances in angiospermic plants[3].

Guduchi [*Tinospora cordifolia* (Willd.) Hook. F. Thoms] is a large, glabrous deciduous climbing shrub belonging to the family Menispermaceae[8]. The stem of *Tinospora cordifolia* is rather succulent with long filiform fleshy aerial roots which arise from the branches. The bark is creamy white to grey. The leaves are membranous and cordate. The flowers are small and yellow or greenish yellow. In auxiliary and terminal racemes or racemose panicles, the male flowers are clustered and female are usually solitary. The drupes are ovoid, glossy, succulent, red and pea sized. The seeds are curved. Fruits are fleshy and single seeded. Flowers grow during the summer and fruits during the winter[5].

The development of plant cell cultures, nowadays, is an important strategy for bio prospection of natural products. Thus the large scale production of bioactive compounds or extracts used as phytotherapics,

pharmacological products, food additives and cosmetics should be encouraged because of their scientific, economical or ecological importance (Bourgaud *et al.*,). However the production of bioactive metabolites can be regarded as the result of the interaction of environmental conditions and genotype of the cultured plant cells. Thus, culture medium and their constituents, light and temperature control the metabolism and the growth of callus. Present investigation analyzes the maintenance of antibacterial activity in extracts *in vivo* and *in vitro.*

MATERIALS AND METHODS

Collection of plants

The young leaves and stem of *Tinospora cordifolia* were collected from 3-5 months old healthy plant grown in Karaikudi, Tamil Nadu, India, that receives a mean annual rainfall ranging from 100 to 120 cm with an average temperature of 38˚C. The pH of the garden soil was 7. The plant leaves and stem were washed thoroughly with tap water followed by sterile distilled water and shade dried at room temperature for 10-15 days.

Media Preparation, Callus Initiation and its Proliferation

MS media[7] supplemented with auxins *viz*., 2,4-D, NAA, BAP and cytokinins *viz*., BAP and Kn alone at different concentrations was used for callus induction. The cultures were maintained in a culture room at 16/8 h light/dark conditions by using cool white fluorescent tubes (40 μM m^{-2} s^{-1}) with 55-60% relative humidity. Each treatment had 10-25 replicates and was repeated thrice.

Collection and storage of field grown plant and callus materials

The plant parts were collected from 3-5 months old mature plants and washed with water and then chopped into small fragments. The materials were then shade dried at ambient temperature (32°C) for 4 - 5 days and the drying operation was carried out under controlled conditions to avoid chemical changes. The dried samples were crushed into fine powder using an electronic blender. The powdered samples were stored in polythene containers at room temperature. The callus material obtained from various explants were collected at the end of four week and dried in hot air oven at 50°C for 48 hours. Then the dried material was powdered using mortar and pestle and the powdered samples were stored in polythene containers at room temperature**.**

Preparation of extracts

The organic constituents from dried plant (Leaf & Stem) material were obtained by continuously extracting the powdered material in soxhlet apparatus with ethanol: water (4:1) as organic solvent for 24 hours at 55°C until complete exhaustion of the material. After completion of extraction, the extracts were passed through Whatman No.1 filter paper and the

filtrate was concentrated in vacuum rotary evaporator at 60°C in order to reduce the volume. The paste like extracts were stored in labelled screw capped bottles and kept in refrigerator at 4°C [9]. Likewise extracts were also prepared using the solvent Chloroform: water (4:1) and the aqueous extract was prepared using distilled water and extracted at 100 °C.

Microorganisms used

The fungal strains included *Aspergillus niger, A. flavus, A. fumigatus, A. oryzae, Epidermophyton floccosum, Madura mycetoma, Trichophyton mentagrophyte, T. rubrum* and *T. tonsurans.*

Antifungal assay

Poisoned food technique (Nene, 1970)

The antifungal activity of the plant extract was assayed by the poisoned food technique. Here the inoculum was taken in the form of a disc from non-sporulating mycelium and placed at the centre of PDA medium in petridish. The diameter of the mycelial growth indicates the antifungal effect of the extract. Extract of leaf and leaf derived callus of *T. cordifolia* was incorporated into the sterile medium in required quantities. Plates of PDA without extract were also inoculated with fungal discs to serve as control. An inoculum of 6 mm diameter disc of non-sporulaing mycelium was cut from the edge of actively growing fungal colony using a cork borer and placed at the centre of the agar medium in a petridish. The experiments were conducted in triplicate along with equal number of controls.

The plates were incubated at 27°± 1 C and the diameter of the fungal colonies was measured at 24 h intervals and the mean of the diameter of growth was recorded. 1 mg/ml concentration of the extract was tested for the activity. The percentage of inhibition was calculated by the formula.

$$I = \frac{[(C - T) \times 100]}{C}$$

Where, I = percentage inhibition of fungal growth
C = diameter of fungal growth in control
T = diameter of fungal growth in test.

RESULTS

Antifungal activity test of the ethanol extracts of field grown leaves and leaf derived callus was performed with different fungal sp. The fungal strains included *Aspergillus niger, A.flavus, A. fumigatus, A. oryzae, Candida albicans, Epidermophyton floccosum, Madura mycetoma, Trichophyton mentagrophyte, T. rubrum* and *T. tonsurans.* The antifungal activity was assessed using poisoned food technique. The antifungal activity was higher in leaf derived callus extract than in natural leaf extract (Table 1). The higher antifungal activity (55% inhibition) of leaf derived callus extract was found against *Aspergillus oryzae* while the lower activity (30% inhibition) was found against *T. mentagrophyte*.

DISCUSSION

A variety of compounds with antifungal activity are present in plants[1]. The search for simple bioactive compounds of plant origin against fungi has been the topic of interest for ecologically safe products. In the present study, ethanolic extracts of *Tinospora cordifolia* leaf showed maximum antifungal activity against *Candida albicans* and *Aspergillus* sp., this activity is attributed to its berberine content[11]. These results are supported by the findings of Freile *et al.,* (2003) who reported that methanolic extracts of *Berberis actensis* root showed good activity against *Candida albicans, C. krusei and C. tropicalis* but not against *C. parapsilosis.* Supporting the present study Iauk *et al.,* (2007) stated significant antifungal activity of berberine against different isolates of *Candida* sp.

Ethanolic extracts of *Tinospora cordifolia* were active against all tested *Aspergillus* sp. and *Trichophyton* sp. This result is similar to the reports of Lohombo-ekomba *et al.*[6] in *Albertisia villosa* (Menispermaceae). Similar reports of sensitivity of *Aspergillus* sp. to extracts of *T. cordifolia* leaf have been reported by Natarajan and Francis Xavier[9]. Among the tested extracts callus extracts of leaf and stem showed maximum inhibitory activity when compared to the natural leaf and stem extracts. Similar reports of stem derived callus extract showing maximum antifungal activity against *A. niger* and *A. flavus* compared to natural stem extract has been reported by Kaviraj *et al.,*[4] in *Arbus precatorius*.

Table – 1: Antifungal activity of callus and field grown extracts of *Tinospora cordifolia* (diameter of fungal growth in cm)

S.No	Name of the fungi	Control	Natural leaf extract	Leaf derived callus extract	Percent inhibition of solvent	Percent inhibition of growth by natural leaf extract	Percent inhibition of growth by leaf callus extract
1.	*Aspergillus flavus*	2.0	1.5	1.1	1.0	15.8 ± 0.28^{c}	35.4 ± 0.82^{ef}
2.	*A. fumigatus*	1.8	1.5	1.2	1.50	9.8 ± 0.36	35.5 ± 0.64
3.	*A. niger*	4.0	1.6	1.3	12.5	46.98 ± 0.30	55.0 ± 0.5
4.	*A. oryzae*	2.5	1.8	1.2	12.0	15.80 ± 3.6	41.2 ± 1.5^{ef}
5.	*Epidermophyton floccosum*	2.3	2.1	1.3	17.8	17.18 ± 3.2^{b}	31.9 ± 0.42
6.	*Madura mycetoma*	2.9	2.0	0.9	33.0	26.1 ± 0.08	29.78 ± 0.40
7.	*T. mentagrophyte*	2.6	2.0	1.2	11.0	12.8 ± 0.001	42.8 ± 0.32^{f}
8.	*T. rubrum*	2.4	1.8	1.0	8.30	16.81 ± 0.08	49.72 ± 0.68
9.	*T. tonsurans*	2.7	2.0	0.9	7.40	17.62 ± 0.32	58.62 ± 0.35

Values are mean of three replicates. Means followed by same letters are statistically not significant at $\alpha = 0.05$ by Duncan's multiple range test.

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