Journal of Applied Pharmaceutical Science Vol. 3 (08), pp. 041-045, August, 2013
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2013.3808
ISSN 2231-3354

Comparison of antifungal activity of medicinal plant *Tylophora indica* Merr. with its *in vitro* raised plant and callus

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ARTICLE INFO

Article history:
Received on: 06/07/2013
Revised on: 22/07/2013
Accepted on: 05/08/2013
Available online: 30/08/2013

Key words:
Tylophora indica, in vitro raised callus, antifungal activity, agar well diffusion, minimum inhibitory concentration.

ABSTRACT

The antifungal potential of medicinal plant *Tylophora indica* Merr. was evaluated by agar well diffusion method and it was compared with the activity of its *in vitro* raised plant and callus . The extracts of *in vitro* raised plant and callus showed better antifungal activity against the tested fungal species as compared to parent plant. Minimum inhibitory concentrations (MIC) of the extracts were determined by broth microdilution method. The MIC of the alcoholic leaf extracts of parent plant against tested fungi ranged from 12.0 to 98.0 µg/ml, whereas, the MIC of extract of *in vitro* raised plant and callus ranged from 1.53 to 49.0 µg/ml and 3.05 to 24.0 µg/ml respectively. The present study leads to conclusion that extracts of *Tylophora indica* contain good antifungal activity which could be used in the treatment of various fungal infections showing resistance to treatment by currently used antifungal agents. As the *in vitro* raised plant and callus gave good results, *in vitro* cultivation of the explants may be used to obtain novel antifungal compounds. This is the first report on antifungal activity of *Tylophora indica* through *in vitro* raised plant and its callus.

INTRODUCTION

Opportunistic fungal infections are increasing as a consequence of the unprecedented increase in numbers of immunocompromised patients from various areas of the health care system. The situation has become even more alarming with the current pandemic of AIDS. Due to increasing development of drug resistance in human pathogens as well as the appearance of undesirable effects of certain antifungal agents, there is a need to search for new agents (Phongpaichit et al., 2005). A vast majority of synthetic antibiotics controls the growth and development of microorganisms effectively, but they are highly toxic at their optimum dosage level. Among many proposed strategies, a good understanding of plants offers the potential of developing potent broad spectrum antibiotics (Reddy, 2009). One of the medicinal plants is *Tylophora indica* Merr. (Asclepiadaceae), also known as 'Indian ipecac' in English, 'Jangali pikvan' in Hindi and 'Anntmool' in Sanskrit. It is a dark copper coloured delicate creeper found growing wild in the plains of India and other sub-tropical regions of the world (Bhavan, 1992). The plant inhabits up to an elevation of 1,260 m in the sub-Himalayan tract and in the central and peninsular India. It also grows in Eastern, North-East and Central India, Bengal and, parts of South India (Nadkarni, 1976) and parts of Ceylon, Malay island and Borneo (Kirtikar and Basu, 1935).

It is a perrineal, small, slender, much branched pubescent twining or climbing herbs or under shrubs. Leaves 6.0-10.5 × 3.8-6.0 cm, ovate-oblong to elliptic-oblong, petioles are up to 12 mm long. Flowers are minute, 1-1.5 cm across, in 2 to 3-flowered fascicles in axillary umbellate cymes. Fruits are up to 7×1 cm; ovoid-lanceolate, tapering at apex. Flowers and fruits are produced between August-December (Kirtikar and Basu, 1935; Chopra et al., 1956a,b).

It is traditionally used as a folk medicine in certain regions of India for the treatment of bronchial asthma (Bielory and Lupoli, 1999), inflammation, bronchitis, allergies, and dermatitis (Gupta and Bal, 1956; Dhananjayan et al., 1974; Thiruvengadam et al., 1978; Gupta et al., 1979; Gore et al., 1980).

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It is regarded as one of the best indigenous substitute for ipecacuanha, so it was considered as Indian ipecacuanha in the latter half of the 19th century (Kirtikar and Basu, 1935). The leaves are employed to destroy vermin. The leaf extracts also act as anti tumour agents (Chitnis et al., 1972; Stephen and Vijayammal, 2000). It has reputation as an alternative blood purifier and has often been used in rheumatism. The roots and leaf powder are used in diarrhea, dysentery and intermittent fever. It was also identified as a good remedy in traditional medicine for psoriasis, anaphylaxis and leucopenia (Sangeetha et al., 2012).

But unfortunately, these plants are disappearing at an alarming rate due to indiscriminate deforestation and uncontrolled collection of plant materials (Vanila et al., 2008). Through *in vitro* cultivation it would be possible to preserve and conserve these endangered plants and obtain phytotherapeutic compounds especially at places where the plant does not grow naturally due to adverse atmospheric conditions (Shahid et al., 2009b).

Although *T. indica* is a versatile medicinal plant, with its use being restricted in localities of Indian sub continents and parts of Africa, the information on the antimicrobial and antifungal activity of *Tylophora* species is insufficient. Hence the present study was carried out to evaluate the antifungal potential of medicinal plant *Tylophora indica* Merr. and to compare its activity with its *in vitro* raised plant extract and callus.

MATERIALS AND METHODS

Collection of plant materials

Fresh leaves were collected from 6 years old plant of *Tylophora indica* (Figure 1A) grown in the Botanical garden, Department of Botany, Aligarh Muslim University, Aligarh.

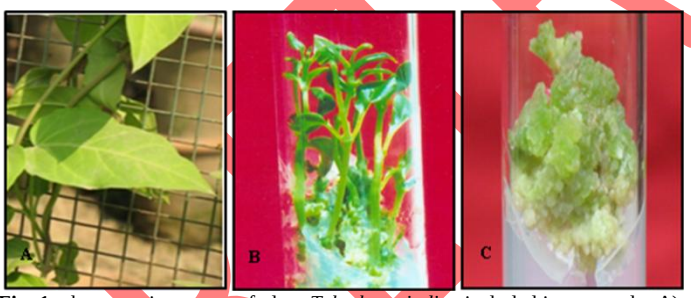

Fig. 1: shows various parts of plant *Tylophora indica* included in our study. A) Leaf of parent palnt, B) Leaf of in vitro raised plant and C) Leaf callus.

IN VITRO CULTURE OF EXPLANTS

In vitro shoot regeneration

The leaf explants were cultured on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) containing 5µM of BA [6-Benzyladenine]. Callus formation started after 4 weeks of incubation and shoot bud induction took place in 6 weeks. Shoot buds transformed into elongated shoots after second subculture passage in the fresh medium of same composition. These microshoots (3-5 cm long) were transferred to root induction medium containing MS + IBA [Indole 3-Butyric Acid] (2.5µM). Healthy roots were induced within 2 weeks of transfer. The rooted plantlets were acclimatized initially in culture room conditions by transferring in soilrite containing thermocole cups. After one month hardening these were transferred to green house conditions. The plants thus obtained were then used further for antimicrobial studies using its leaves (Figure 1B).

In vitro induction of Leaf callus

The leaf explants were cultivated in callus induction medium comprising of 5µM of MS + 2,4-D[2,4-Dichlorophenoxy acetic acid]. Callus was initiated from the cut ends of the explants after 25 days of inoculation. 4g fresh weight of callus (dry weight of 367 mg) was induced after 5 weeks of culture which was used for evaluation of antifungal effect (Figure 1C).

Plant extracts

The alcoholic extracts of the plant were tested for antimicrobial activity. The extracts were derived according to the method of Singh and Singh (2000) with some modifications (Shahid et al., 2007, 2009a,b). The extracts were prepared by taking fresh leaves (15 g) from both sources (*in vivo* and *in vitro*) and sterilizing in 70% ethyl alcohol for 1 min. These were then washed 3 times with sterilized double distilled water (DDW). The leaf calli were aseptically removed from the culture tubes and all the plant materials, including calli, were grounded with a sterile pestle and mortar in 150 ml of absolute alcohol. The homogenized tissues were centrifuged at 5000 rpm for 15 min, and the supernatant was filtered and taken as the alcoholic extracts which were immediately used for experimentation.

Fungi tested

The clinical fungal strains included in our study were *Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* spp. and *Fusarium* spp., isolated in the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India. The control strains tested were *C. krusei* (ATCC 6258) and *A. fumigatus* (ATCC 204305) obtained from New Drug Discovery Research, Ranbaxy, Gurgaon, India. The fungi were grown at 25°C in biological oxygen demand (BOD) incubator and maintained on Sabouraud's Dextrose agar (SDA) slants.

Antifungal susceptibility testing

Antifungal activity was determined on SDA plates using agar well diffusion method (Vanden-Berghe and Vlietinck, 1991; Akinpelu, 2001), with some modifications (Shahid et al., 2007). An inoculum size of 2×10^6 yeast cells or fungal spores was used for inoculating the susceptibility plates. These plates were lawn cultured with fungal suspensions with the help of sterile swabs and wells of 5mm diameter were made in each plate using a sterile borer. Plant extracts (20µl) were poured in the wells using micropipette. 20µl of 95% ethanol was used to serve as negative control, whereas, antifungal agent voriconazole (500µg/20µl) was used as positive control. The plates were kept upright for 5-10 min

until the solution diffused into the medium and then incubated aerobically at 25°C in a biological oxygen demand (BOD) incubator for 2-5 days. Later, the zone of inhibition was measured and recorded. All experiments were performed in triplicate.

Determination of minimum inhibitory concentrations (MIC)

MIC was determined by broth micro-dilution method performed according to Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS (1997) for yeasts and NCCLS (2002) for filamentous fungi, with minor modifications (Shahid et al., 2007). Doubling dilutions of the extract was prepared using RPMI-1640 (HiMedia, India) broth supplemented with 0.3g/L L-glutamine (HiMedia, India), 0.165mol/L of 3-[N-morpholino] propanesulfonic acid (MOPS) buffer (HiMedia, India) and 0.01% of Dimethyl sulphoxide (DMSO) (Qualigens Fine Chemicals, India). Extracts were dissolved in DMSO, and further diluted 1:50 in RPMI-1640 medium, and each resulting solution was used for a doubling dilution series. Microtitre plates were prepared containing 100µl of undiluted extracts in the first well, followed by doubling dilutions of extracts. The standardized inoculum of each fungal species was added to the respective dilution wells. The final concentrations of the extracts ranged from 25×10^{3}µg/ml to 48×10^{-3}µg/ml. For each test there was a sterility control well containing alcoholic extract in RPMI-1640 broth plus DMSO and a growth control well containing fungal suspension without alcoholic extract. The microtitre plates were incubated at 35°C for 48 hours with their upper surface covered by sterile sealers. The lowest concentration that did not show any visible growth was considered the MIC of that extract for the tested fungal species. All the MIC experimentations were performed in duplicate.

Statistical analysis

All the experiments of antimicrobial susceptibility testing were performed in triplicate. The results were expressed as the mean ± standard error (SE). Data were analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey's multiple analysis test using SPSS Software, Chicago, III, version 10. P values were calculated by one-sample T-test and $P < 0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Antimicrobial activity of alcoholic extracts of parent plant as well as its *in vitro* raised plant and calli against the tested fungal species is shown in Table 1. Negative control (ethanol) showed the zone of inhibition in the range of 7.33±0.33 to 8.67±0.33 mm. Positive control (voriconazole) showed the zone of inhibition in the range of 9.33±0.33 to 12.67±0.33 mm. The alcoholic leaf extract of parent plant showed significant ($P<0.05$) antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Penicillium* spp. (Table 1). Similar studies done by different workers have detected significant antifungal activity of this plant extract which supports our findings (Reddy et al., 2009; Sangeetha et al., 2012).

The antifungal activity of alcoholic leaf extract of *in vitro* raised plant was found to be better in comparison to the activity of parent plant extract (Table 1). It showed significant activity ($P<0.05$) against *Candida albicans* and *Candida krusei*, in addition to activity against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus* and *Penicillium* spp. This enhanced activity of *in vitro* raised plant could be due to nutritional and hormonal manipulations done in the culture medium. To the best of our knowledge this is the first study analyzing the antifungal potential of *in vitro* raised plant, therefore, our findings could not be compared.

The *in vitro* raised leaf callus also showed good antifungal activity which was comparable to the activity shown by *in vitro* raised plant (Table 1). The alcoholic extract of *in vitro* cultivated leaf callus showed significant activity against *Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. To the best of our knowledge this is the first study analyzing the antifungal potential of *in vitro* raised callus of this plant, therefore, our findings could not be compared.

The MIC of the alcoholic leaf extracts of parent plant against tested fungi ranged from 12.0 to 98.0 µg/ml (Figure 2), whereas, the MIC of *in vitro* raised plant extract and callus extract ranged from 1.53 to 49.0 µg/ml and 3.05 to 24.0 µg/ml respectively (Figures 3 and 4).

Table. 1: Antifungal activity of alcoholic extracts of parent plant of *Tylophora indica* and its *in vitro* raised plant and leaf callus against pathogenic and opportunistic Fungi.

Fungi tested	Zone of inhibition (mm) ± SE				
	Alcoholic leaf extract of parent plant$^{\Delta}$	Alcoholic leaf extract of *in vitro* raised plant$^{\Delta}$	Alcoholic extract of leaf callus$^{\Delta}$	Ethanol† (negative control)	Voriconazole$^{£}$ (positive control)
Candida albicans	0.00±0.00^{e}	14.67±0.33^{ab}	12.67±0.33^{b}	8.67±0.33^{a}	12.67±0.33^{a}
Candida krusei	0.00±0.00^{e}	12.67±0.33^{c}	12.33±0.33^{bc}	8.33±0.33^{b}	11.00±0.58^{bc}
Aspergillus fumigatus	11.33±0.33^{b}	13.00±0.58^{bc}	11.67±0.33^{c}	7.33±0.33^{d}	10.33±0.33^{d}
Aspergillus flavus	10.67±0.33^{c}	11.67±0.33^{d}	10.33±0.33^{e}	7.67±0.33^{c}	9.67±0.33^{e}
Aspergillus niger	10.33±0.33^{d}	11.33±0.33^{e}	11.00±0.58^{d}	7.33±0.33^{d}	9.33±0.33^{f}
Penicillium spp.	13.00±0.58^{a}	15.33±0.67^{a}	0.00±0.00^{f}	8.33±0.33^{b}	11.67±0.33^{b}
Fusarium spp.	0.00±0.00^{e}	0.00±0.00^{f}	0.00±0.00^{f}	7.33±0.33^{d}	9.33±0.33^{f}
C. krusei (ATCC 6258)	0.00±0.00^{e}	13.67±0.33^{b}	13.33±0.33^{a}	8.67±0.33^{a}	12.33±0.33^{ab}
A. fumigatus (ATCC 204305)	12.33±0.33^{ab}	13.00±0.58^{bc}	11.00±0.58^{d}	7.67±0.33^{c}	10.67±0.33^{c}

† = 20 µl of 95% ethanol was used as negative control. Δ = concentration of extracts used in the test i.e. 2 mg / 20 µl. £ = concentration of voriconazole used in test i.e. 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. $P<0.05$ was considered as significant. The mean represented by same letter is not significantly different within the column.

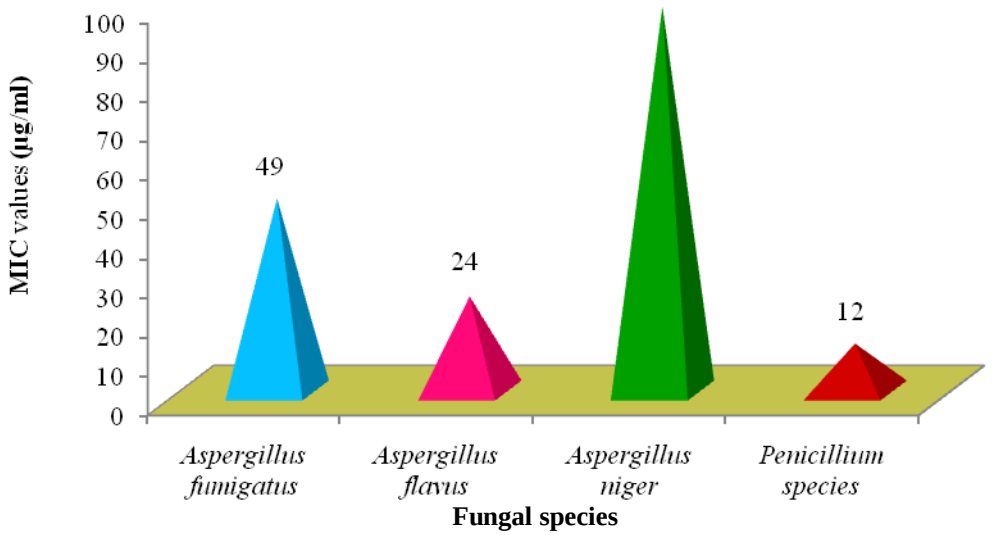

Fig. 2: MIC determination of alcoholic leaf extract of parent plant of *Tylophora indica* against tested fungal species.

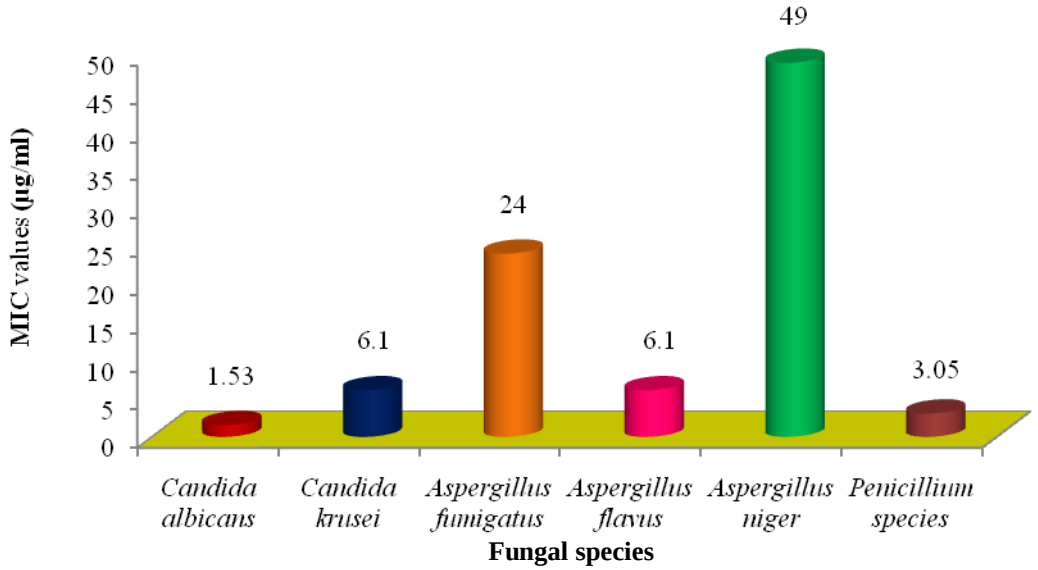

Fig. 3: MIC determination of alcoholic leaf extract of in vitro raised plant of *Tylophora indica* against tested fungal species.

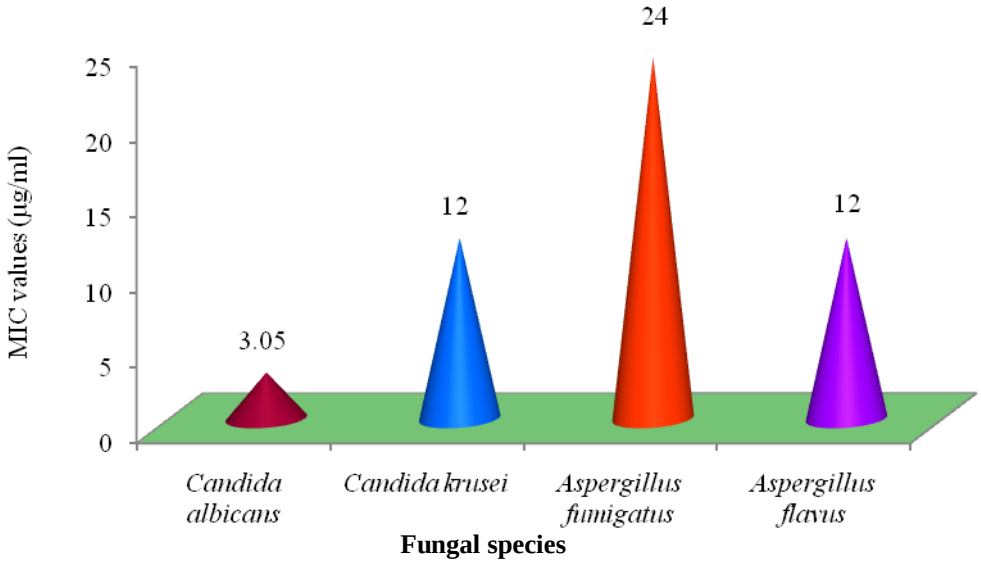

Fig. 4: determination of alcoholic extract of leaf callus of *Tylophora indica* against tested fungal species.

CONCLUSION

To conclude, the alcoholic leaf extract from *in vitro* raised plant of *T. indica* showed better antifungal activity as compared to parent plant extract. As the alcoholic extract of *in vitro* raised plant and callus showed good activity against *Candida albicans* and *Candida krusei*, which was not detected in the extract of parent plant, hence, *in vitro* raised plant extract could be used in the treatment of infectious diseases caused by these organisms. The high antifungal activity of *in vitro* raised plant and callus may be due to nutritional and hormonal manipulations in the cultivation medium. This shows the future prospect of these extracts to be used as novel antifungal agents and *in vitro* cultivation may be used to obtain phytotherapeutic compounds, especially at places where this plant does not grow naturally because of adverse atmospheric conditions.

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How to cite this article:

Razia Khatoon, Noor Jahan, Anwar Shahzad, Mohammad Shahid., Comparison of antifungal activity of medicinal plant *Tylophora indica* Merr. with its *in vitro* raised plant and callus. J App Pharm Sci, 2013; 3 (08): 041-045.