Comparison of antibacterial activity of parent plant of *Tylophora indica* Merr. with its *in vitro* raised plant and leaf callus

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The antibacterial potential of an endangered medicinal plant *Tylophora indica* was analyzed by agar well diffusion method and its activity was compared with that of its *in vitro* raised plant and callus. The extracts of parent plant of *T. indica* showed good antibacterial activity against gram negative bacteria only; whereas, the extracts from *in vitro* raised plant and leaf callus showed good activity against both gram positive and gram negative bacteria. Minimum Inhibitory Concentration (MIC) of the alcoholic leaf extract of *in vitro* raised plant was determined by broth microdilution method. MIC against gram positive bacteria ranged from 3.05 to 12.0 µg/ml and MIC against gram negative bacteria ranged from 1.53 to 24.0 µg/ml. The present study leads to conclusion that extracts of *T. indica* contains good antibacterial activity which can be used in the treatment of various infections showing resistance to treatment by currently used antimicrobial agents. As the *in vitro* raised plant and callus gave better results as compared to parent plant, *in vitro* cultivation of explants may be used to obtain novel antibacterial compounds. This is the first report on antibacterial activity of *T. indica* through *in vitro* raised plant and its callus.

**Key words:** *Tylophora indica, in vitro* raised plant and callus, antibacterial activity.

**INTRODUCTION**

Infectious diseases account for high proportion of health problems and are the leading cause of death worldwide (Parekh and Chanda, 2007a). Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents and their resistance to old and newly produced drugs is on rise (Sangeetha et al., 2012). This is due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. The global emergence of multidrug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Davies, 1994; Hancock, 2012).
Although, T. indica is a versatile medicinal plant, with its use being restricted in localities of Indian sub continent and parts of Africa; the information on the antimicrobial activity of Tylophora species is insufficient. Hence, the present study was carried out to evaluate the antibacterial potential of medicinal plant T. indica Merr. and compare its activity with its in vitro raised plant extract and callus.

**MATERIALS AND METHODS**

**Collection of plant materials**

Fresh leaves were collected from six years old plant of T. indica grown in the Botanical garden, Department of Botany, Aligarh Muslim University, Aligarh.

**In vitro culture of explants**

**In vitro shoot regeneration (for in vitro plant extract)**

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

**In vitro induction of leaf callus**

The leaf explants were cultivated in callus induction medium comprising of MS + 5 µM of 2,4-D (2,4-dichlorophenoxy acetic acid). Callusing was initiated from the cut ends of the explants after 25 days of inoculation. Callus was yellow in colour and friable in nature. 4 g fresh weight of callus was induced after 5 weeks of culture which was used for evaluation of antimicrobial effect.

**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, 2009b). To prepare alcoholic extracts, fresh leaves (15 g) from both sources (parent plant and in vitro raised plant) were surface sterilized in 70% ethyl alcohol for 1 min and 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 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 extract. The extracts were immediately used for experimentation.

Microorganisms tested

The clinical bacterial strains included in our study were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae* type 1 and *Vibrio cholerae* isolated from clinical specimens in the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India. The control strains tested were *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) obtained from National Institute for Communicable Diseases (NICD), New Delhi, India. These strains were grown on Blood agar or MacConkey agar plates at 37°C and maintained on nutrient and blood agar slants.

Antibacterial susceptibility testing

Antibacterial activity was determined using agar well diffusion method (Vanden-Berghe and Vlietinck, 1991; Akinpelu, 2001), with some modifications (Shahid et al., 2007). Antibacterial tests were performed as per Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards (2000) using Mueller-Hinton Agar (M 173; HiMedia, India). For fastidious organisms such as *Streptococci*, 5% sheep blood agar was used. An inoculum containing $10^6$ cfu/ml of bacteria was used for inoculating the susceptibility plates. The plates were lawn cultured with the bacterial suspensions with the help of sterile swabs and wells of 5 mm diameter were made in each plate using a sterile borer. Plant extracts (20 µl) were poured in the wells using micro-pipette. 20 µl of 95% ethanol was used to serve as negative control, whereas, antibacterial agent gentamicin (500 µg/20 µl) was used as positive control. The plates were kept upright for 5 to 10 min until the solution diffused into the medium and then incubated aerobically at 37°C for 24 h. 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 (formerly National Committee for Clinical Laboratory Standards, 2000), with some modifications (Shahid et al., 2007). Doubling dilutions of the extracts were prepared using RPMI-1640 broth (HiMedia, India) supplemented with 0.3 g/L L-glutamine (HiMedia, India), 0.165 mol/L of 3-[N-morpholino] propanesulfonic acid (MOPS) buffer (HiMedia, India) and 0.01% of dimethyl sulphoxide (DMSO) (Qualigen Fine Chemicals, India). The 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 100 µl of undiluted extracts in the first well, followed by doubling dilutions of extracts from second well. The standardized inoculum of each bacterial species was added to the respective dilution wells including the first well. The final concentrations of the extracts ranged from $25 \times 10^{-3}$ to $48 \times 10^{-3}$ µg/ml. For each test, there was a sterile control well containing alcoholic extract in RPMI-1640 broth plus DMSO and a growth control well containing bacterial suspension without alcoholic extract. The microtitre plates were incubated at 35 ± 2°C for 24 h 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 bacterial 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 statistically analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple analysis test using SPSS Software, Chicago, Ill, 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 leaves of parent plant as well as its in vitro raised plant and leaf callus against the tested bacterial species is shown in Tables 1 and 2. Negative control (ethanol) showed the zone of inhibition in the range of 7.33 ± 0.33 to 8.67 ± 0.33 mm. Positive control (gentamicin) showed the zone of inhibition in the range of 9.67 ± 0.33 to 13.00 ± 0.58 mm. All the extracts showed good antibacterial activity. The alcoholic leaf extract of parent plant showed good activity against tested gram negative bacteria only and no activity against tested gram positive bacteria (Tables 1 and 2). It showed significant (P<0.05) activity against *E. coli* (P = 0.024), *K. pneumoniae* (P = 0.020) and *P. aeruginosa* (P = 0.038). Various studies have been undertaken previously by different researchers to analyze the antibacterial potential of parent plant of *T. indica*. A study done by Parekh and Chanda (2008) also showed no activity of alcoholic leaf extract of parent plant of *T. indica* against *S. aureus* and *S. epidermidis*, which supports our present research findings. On the other hand, study done by Reddy (2010) showed significant activity of this plant against *S. aureus*, *K. pneumoniae*, *E. coli*, *S. typhi*, *P. aeruginosa* and *P. vulgaris*. Another study done by Sangeetha et al. (2012) showed significant activity of leaf extract of parent plant of *T. indica* against *S. aureus* only and no activity against *E. coli* and *P. aeruginosa*. These findings are in contrast with our study. This could be due to different concentrations of extracts used in their study as well as variation in active metabolites present in plant extracts derived from different places.

The alcoholic leaf extract of in vitro raised plant of *T. indica* showed good antibacterial activity against most of the tested gram positive bacteria, except *S. pyogenes* and *E. faecalis* (Table 1). It showed significant (P<0.05) activity against *S. aureus* (P = 0.005), *S. epidermidis* (P = 0.003) and *B. subtilis* (P = 0.044), with its MIC ranging from 3.05 to 12.0 µg/ml (Figure 1). The alcoholic leaf extract of in vitro raised plant showed good activity against most of the tested gram negative bacteria (Table 2). It showed significant activity (P<0.05) against *E. coli* (P = 0.003), *K. pneumoniae* (P = 0.012), *P. aeruginosa* (P = 0.010), *S. dysenteriae* type 1 (P = 0.012) and *S. typhi*
(P = 0.038), with its MIC ranging from 1.53 to 24.0 µg/ml (Figure 2). It is interesting to note that these bacteria which are found to be susceptible to the extracts of in vitro raised plant of T. indica are important human pathogens responsible for wound infection, enteric fever, dysentery, urinary tract infection, pneumonia and diarrhea. The in vitro cultivated leaf callus also showed good antibacterial activity which was comparable to the activity shown by in vitro raised plant (Tables 1 and 2). Its alcoholic extract showed significant activity (P < 0.05) against S. aureus and S. epidermidis (Table 1) and most of the tested gram negative bacteria (Table 2). To the best of our knowledge, this is the first study analyzing the antibacterial potential of in vitro raised plant and leaf callus of this plant; therefore, our findings could not be compared.

**Conclusion**

The alcoholic extract of leaves from in vitro raised plant of T. indica and its in vitro cultivated leaf callus showed better antibacterial activity as compared to parent plant extract. These extracts showed wide range of antibacterial activity against various gram negative bacteria as well as against gram positive bacteria like S. aureus and S. epidermidis; hence, they could be used in the treatment of infectious diseases caused by these organisms, which otherwise pose problem of resistance to the commonly used antimicrobial agents. Thus, it leads to a conclusion.
that high antibacterial activity of *in vitro* raised plant and callus may be due to enhancement of the bioactive compounds responsible for antibacterial effects by nutritional and hormonal manipulations in the cultivation medium as depicted in our study. This shows the future prospect of these extracts which can be used as novel antibacterial agents. Also, *in vitro* callus induction may be used to obtain phytotherapeutic compounds, especially at places where this plant does not grow naturally because of adverse atmospheric conditions.