

Full Length Research Paper

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

Noor Jahan<sup>1,2\*</sup>, Razia Khatoun<sup>1,2</sup>, Anwar Shahzad<sup>3</sup>, Mohammad Shahid<sup>1,4</sup> and Siraj Ahmad<sup>5</sup>

<sup>1</sup>Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh-202002, India.

<sup>2</sup>Department of Microbiology, Era's Lucknow Medical College and Hospital, Lucknow-226003, India.

<sup>3</sup>Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh-202002, India.

<sup>4</sup>Department of Medical Microbiology, Immunology and Infectious Diseases, College of Medicine and Medical Sciences, Arabian Gulf University, Manama-26671, Kingdom of Bahrain.

<sup>5</sup>Department of Community Medicine, Teerthanker Mahaveer Medical College and Research Centre, Teerthanker Mahaveer University, Moradabad- 244001, India.

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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,

\*Corresponding author. E-mail: drnoorj@rediffmail.com.

2005). This situation has forced the researchers to search for new antimicrobial substance from various sources including medicinal plants (Scazzocchio et al., 2001; Erdogru, 2002; Bandow et al., 2003; Parekh and Chanda, 2008). Plants are a goldmine of novel chemicals and an impressive number of modern drugs have been developed from them (Reddy, 2010). There are several reports in literature regarding the antimicrobial activity of crude extracts prepared from plants (El-Seedi et al., 2002; Rojas et al., 2003; Duraipandiyar et al., 2006; Parekh and Chanda, 2007b). Antimicrobials of plant origin have proved effective in the treatment of several infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Samy and Ignacimuthu, 2000). There are more than 2,70,000 higher plants existing in this planet. But so far, less than 10% of recorded flora has been explored phytochemically as well as clinical evaluation for various biological activities (Reddy, 2010).

*Tylophora indica* Merr. (Asclepiadaceae) is a medicinal plant indigenous to India. It is also known as 'Indian ipecac' in English, 'Jangali pikvan' in Hindi and 'Anntmool' and 'Anthrachaka' in Sanskrit, 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 (Nadkarni, 1976). It is also found in 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 are ovate-oblong to elliptic-oblong. Flowers are minute, 1 to 1.5 cm across. Fruits are up to 7 × 1 cm, ovoid-lanceolate and tapering at apex. Flowers and fruits are produced between August to December (Kirtikar and Basu, 1935; Chopra et al., 1956a, 1956b). 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, rheumatism and dermatitis (Gupta and Bal, 1956; Dhananjayan et al., 1974; Gore et al., 1980). The roots and leaves are also reported to be used in hydrophobia. Dried leaves are emetic diaphoretic and expectorant. 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 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 soilrite 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 micropipette. 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 minor 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) (Qualigens 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 sterility 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  $35 \pm 2^\circ\text{C}$  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  $\pm$  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  $7.33 \pm 0.33$  to  $8.67 \pm 0.33$  mm. Positive control (gentamicin) showed the zone of inhibition in the range of  $9.67 \pm 0.33$  to  $13.00 \pm 0.58$  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*

**Table 1.** Antibacterial activity of alcoholic extracts of parent plant of *Tylophora indica* and its *in vitro* raised plant and leaf callus against pathogenic gram-positive bacteria.

Bacteria tested	Zone of inhibition (mm) ± SE				
	Alcoholic leaf extract of parent plant <sup>Δ</sup>	Alcoholic leaf extract of <i>in vitro</i> raised plant <sup>Δ</sup>	Alcoholic extract of leaf callus <sup>Δ</sup>	Ethanol <sup>†</sup> (negative control)	Gentamicin <sup>£</sup> (positive control)
<i>Staphylococcus aureus</i>	0.00±0.00 <sup>a</sup>	14.67±0.33 <sup>ab</sup>	13.00±0.58 <sup>b</sup>	7.33±0.33 <sup>d</sup>	12.67±0.33 <sup>b</sup>
<i>Staphylococcus epidermidis</i>	0.00±0.00 <sup>a</sup>	15.33±0.67 <sup>a</sup>	12.67±0.33 <sup>c</sup>	8.33±0.33 <sup>b</sup>	12.33±0.33 <sup>c</sup>
<i>Streptococcus pyogenes</i>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>d</sup>	0.00±0.00 <sup>d</sup>	8.67±0.33 <sup>a</sup>	13.00±0.58 <sup>a</sup>
<i>Enterococcus faecalis</i>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>d</sup>	0.00±0.00 <sup>d</sup>	7.67±0.33 <sup>c</sup>	9.67±0.33 <sup>d</sup>
<i>Bacillus subtilis</i>	0.00±0.00 <sup>a</sup>	13.00±0.58 <sup>c</sup>	0.00±0.00 <sup>d</sup>	7.33±0.33 <sup>d</sup>	12.33±0.33 <sup>c</sup>
<i>S. aureus</i> ATCC 25923	0.00±0.00 <sup>a</sup>	14.33±0.33 <sup>b</sup>	13.67±0.33 <sup>a</sup>	8.33±0.33 <sup>b</sup>	13.00±0.58 <sup>a</sup>

† = 20 µl of 95% ethanol used as negative control; Δ = concentration of extracts used in the test that is, 2 mg / 20 µl; £ = concentration of gentamicin used in test that is, 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.

**Table 2.** Antibacterial activity of alcoholic extracts of parent plant of *Tylophora indica* and its *in vitro* raised plant and leaf callus against pathogenic gram-negative bacteria.

Bacteria tested	Zone of inhibition (mm) ± SE				
	Alcoholic leaf extract of parent plant <sup>Δ</sup>	Alcoholic leaf extract of <i>in vitro</i> raised plant <sup>Δ</sup>	Alcoholic extract of leaf callus <sup>Δ</sup>	Ethanol <sup>†</sup> (negative control)	Gentamicin <sup>£</sup> (positive control)
<i>Escherichia coli</i>	12.67±0.33 <sup>b</sup>	14.33±0.33 <sup>ab</sup>	11.33±0.33 <sup>c</sup>	7.67±0.33 <sup>c</sup>	11.67±0.33 <sup>b</sup>
<i>Klebsiella pneumoniae</i>	11.67±0.33 <sup>c</sup>	13.00±0.58 <sup>b</sup>	11.67±0.33 <sup>b</sup>	7.33±0.33 <sup>d</sup>	10.67±0.33 <sup>d</sup>
<i>Proteus vulgaris</i>	0.00±0.00 <sup>e</sup>	0.00±0.00 <sup>e</sup>	0.00±0.00 <sup>f</sup>	8.33±0.33 <sup>b</sup>	11.67±0.33 <sup>b</sup>
<i>Pseudomonas aeruginosa</i>	11.33±0.33 <sup>d</sup>	12.33±0.33 <sup>c</sup>	11.33±0.33 <sup>c</sup>	7.67±0.33 <sup>c</sup>	10.67±0.33 <sup>d</sup>
<i>Salmonella typhi</i>	0.00±0.00 <sup>e</sup>	11.33±0.33 <sup>d</sup>	10.67±0.33 <sup>d</sup>	7.33±0.33 <sup>d</sup>	10.33±0.33 <sup>e</sup>
<i>Salmonella typhimurium</i>	0.00±0.00 <sup>e</sup>	0.00±0.00 <sup>e</sup>	0.00±0.00 <sup>f</sup>	7.67±0.33 <sup>c</sup>	10.67±0.33 <sup>d</sup>
<i>Shigella dysenteriae</i> type 1	0.00±0.00 <sup>e</sup>	11.67±0.33 <sup>cd</sup>	10.33±0.33 <sup>e</sup>	8.67±0.33 <sup>a</sup>	9.67±0.33 <sup>f</sup>
<i>Vibrio cholerae</i>	0.00±0.00 <sup>e</sup>	0.00±0.00 <sup>e</sup>	0.00±0.00 <sup>f</sup>	7.33±0.33 <sup>d</sup>	10.33±0.33 <sup>e</sup>
<i>E. coli</i> ATCC 25922	13.00±0.58 <sup>a</sup>	14.67±0.33 <sup>a</sup>	12.33±0.33 <sup>a</sup>	8.33±0.33 <sup>b</sup>	12.67±0.33 <sup>a</sup>
<i>P. aeruginosa</i> ATCC 27853	11.67±0.33 <sup>c</sup>	12.67±0.33 <sup>bc</sup>	11.67±0.33 <sup>b</sup>	7.67±0.33 <sup>c</sup>	11.33±0.33 <sup>c</sup>

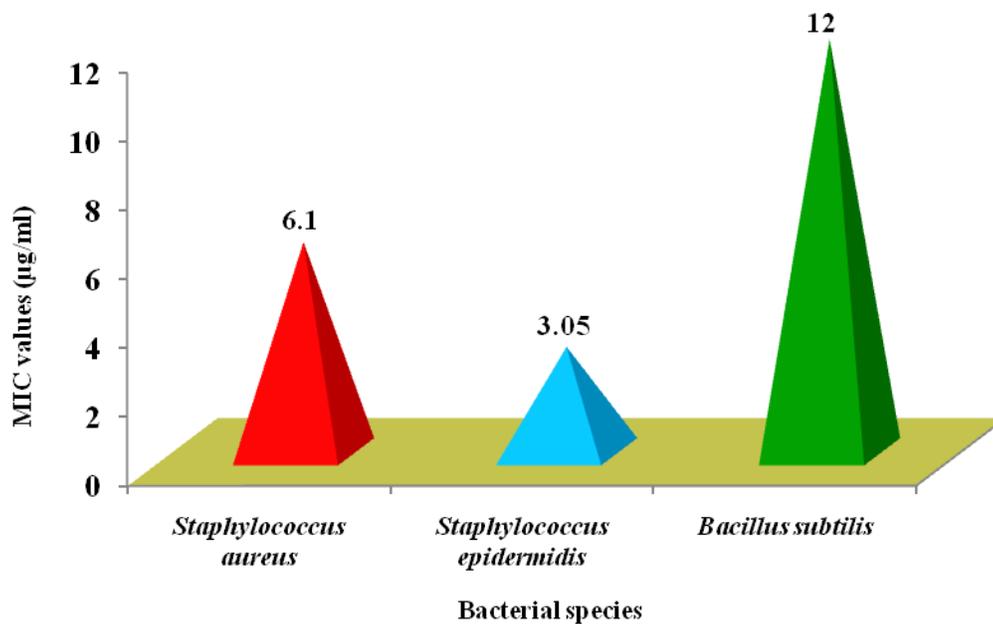
† = 20 µl of 95% ethanol used as negative control; Δ = concentration of extracts used in the test that is, 2 mg / 20 µl; £ = concentration of gentamicin used in test that is, 500 µg / 20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using 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.

(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

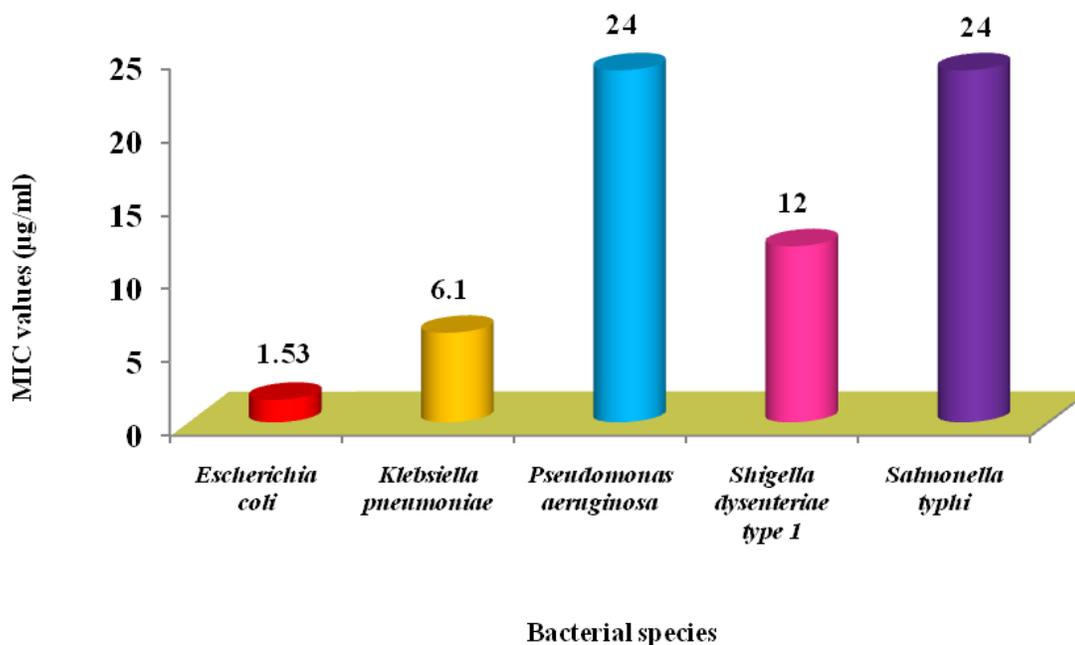
this plant; therefore, our findings could not be compared.

## 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



**Figure 1.** MIC determination of alcoholic leaf extract of *in vitro* raised plant of *Tylophora indica* against tested gram positive bacteria.



**Figure 2.** MIC determination of alcoholic leaf extract of *in vitro* raised plant of *Tylophora indica* against tested gram negative bacteria.

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.

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