African Journal of Biotechnology Vol. 12(9), pp. 958-962, 27 February, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB08.628
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Screening of antimicrobial potential of *in vitro* calli and adult leaf extracts of *Tylophora indica* (Burm. f.) Merril

Sellathurai T.[1], Rathinavel S.[2]* and Natarajan K. K.[2]

[1]Department of Botany, Periyar University, Salem- 636 011, Tamil Nadu, India.
[2]Plant Tissue Culture Lab, Centre for Research in Botany, Saraswathi Narayanan College (Autonomous), Madurai - 625 022, Tamil Nadu, India.

Accepted 23 January, 2009

The analysis of leaf and *in vitro* calli extracts of *Tylophora indica* indicated qualitative difference in the phytochemical compounds. The minimum inhibitory concentration (MIC) of selected solvent extracts of the leaf ranged from 20 to 80 mg/ml. Ethanolic and methanolic extracts were effective in inhibiting most of the tested pathogens but the aqueous extract had poor inhibition. Both the leaf and calli extracts had varying inhibitory action against the test organisms. It may be concluded that *in vitro* calli culture methods could lead to maintain bioactive potential of plants and to formulate antimicrobial drugs of natural origin.

Key words: *Tylophora indica*, leaf, calli, phytocompounds, agar diffusion, pathogens, *in vitro* culture.

INTRODUCTION

In recent years, secondary plant metabolites (phytochemicals) previously with unknown pharmacological activities have been extensively investigated as a source of medicinal agents (Krishnaraju et al., 2005). Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of bacterial infections (Balandrin et al., 1985). Moreover, *in vitro* culture technique played a vital role in mass propagation of valuable medicinal plants towards conservation and sustainable utilization of them to meet the demands of pharmacological industries (Debnath et al., 2006). In this context, *Tylophora indica* (Burm.f.) Merril., a member of Asclepiadaceae is an important indigenous medicinal plant found in restricted localities of India and subcontinent (Chandrasekar et al., 2006). *T. indica* is a perennial climber, shrub up to 1.5 m with long and fleshy roots. Stems are elongate and glabrous but not much branched. Leaves are elliptic, oblong and acute at the top. Flowers are in umbel shape, peduncle arises between the petiole (Rastogi and Mehrotra, 1998). The plant has been scientifically proven for various activities including anti-asthmatic, in allergic rhinitis, as an emetic, smooth muscle relaxant, antihistamic, hypotensive anti-inflammatory, analgesic, anticonvulsant and as anti-rheumatic (Patel et al., 2006). The powdered leaves, stem and root contain several alkaloids including tylophorine ($C_{24}H_{27}O_4N$) and tylophorinine ($C_{23}H_{25}O_4N$) which are pharmacologically active (Chandrasekar et al., 2006). The present work was aimed to investigate antimicrobial property of leaf and *in vitro* cultured calli against selected infectious microorganisms.

*Corresponding author. E-mail: rathinavel_sn@yahoo.com. Tel: 91452 2690635. Fax: 91452 2690505.

MATERIALS AND METHODS

Plant materials

Plants of *T. indica* were collected from the different localities near Madurai and maintained in herbal garden of Saraswathi Narayanan College, Madurai, Tamilnadu, India. The plant was identified by referring to standard taxonomic characteristic features (Keys) according to the flora of Madras Presidency (Gamble, 1935). The fresh materials and healthy leaves of *T. indica* collected during the month of September to October 2007 from herbal garden were used for preparation of extracts.

Callus culture

Healthy shoots were collected from one year old garden plant and washed thrice in tap water. Leaves, stems and shoot tips were cut aseptically and washed with sterilized distilled water for at least 30

min followed by soaking in 5% (v/v) teepol for 5 min. Then subsequently rinsed with distilled water and then with 70% alcohol. The explants were disinfected by immersing in 0.1% (w/v) mercuric chloride solution for 5 min and rinsed thrice with sterilized distilled water on laminar air flow bench. The Murashige and Skoog (1962) medium was gelled with 0.8% (w/v) Agar Type -1 (Himedia). The pH of the medium was adjusted to 5.8 with 0.5 N Hcl or NaOH after incorporation of all the ingredients and various concentrations and combinations of plant growth regulators (Chandrasekar et al., 2006). The different types of calli initiated in the combinations such as MS + BA (2µM), MS + BA (4 µM) and MS + BA (5 µM) + Kin (1 µM) were subcultured to fresh medium. After 3 weeks of interval calli were harvested and used for further studies.

Routinely, 100 ml medium was dispersed in each conical flasks (250 ml) and 20 ml medium in each culture tube (25 × 150 mm). Flasks and tubes were plugged with nonabsorbent cotton and autoclaved for 20 and 15 min respectively at 121°C (1.05 kg cm^{-2}). Sterilized explants were cut into pieces of about 1 cm^2 and then aseptically transferred on to each flask and tube. All cultures were maintained in the culture room at 26.0 ± 0.5°C with 16 h light/8 h dark photoperiods under white fluorescent light (Philips cool TL 36 w: 220 v; light intensity 36 µ mol. M^{-2} S^{-1} and 55-60% relative humidity).

Preparation of extracts

Fresh leaves were washed thoroughly under running tap water, shade dried and used for extraction. Four week old-calli derived from the leaf cultures were collected and dried in an oven at 50 ± 1°C for 60 to 72 h. Both dried leaf and calli were homogenized to a fine powder and stored in air tight bottles. 200 g of leaf powder and 20 gm of calli powder were extracted with 500 and 100 ml of solvents (petroleum ether, chloroform, ethanol, methanol and water) respectively for 24 h using Soxhlet apparatus. The extract was centrifuged *in vacuo* at 40°C evaporator for 30 min and the left over powder was considered 100%. It was stored at 4°C in air tight bottles for antimicrobial assay.

Phytochemical analysis

The dried extracts of leaf and callus were subjected to standard phytochemical analyses for the presence of alkaloids, carbohydrates, tannins, saponins, steroids, fixed oils, fats and phenolic compounds (Harborne, 1973).

Test microorganisms

For the determination of antimicrobial activity, a total of eleven microorganisms including *Staphylococcus aureus*, *Streptococcus faecalis*, *Micrococcus luteus*, *Bacillus subtilis* (Gram positive bacteria); *Escherichia coli*, *Salmonella typhi*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Enterobacter aerogenes* (Gram negative bacteria) and a fungus, *Candida albicans* were obtained from culture collection of the Department of Pharmacology, Madurai Medical College, Madurai, Tamilnadu, India. All the test bacterial species were maintained in nutrient agar media. Thirty six hours old bacterial culture were inoculated into nutrient broth and incubated at 35 ± 2°C on a rotary shaker (Remi, India) at 100 rpm. After 36 h incubation, the bacterial suspension was centrifuged at 10,000 rpm for 15 min. The pellet was resuspended in sterile distilled water and the concentration was adjusted to 1 x 10^8 cfu/ml using UV visible spectrophotometer (Hitachi UV – 2000, Japan) by reading the OD of the solution to 0.45 (A_{610nm}) and used for tests. Fungal colonies were harvested from 9-10 day old cultures which were maintained on potato dextrose agar. The spores were suspended in sterile distilled water and the spore suspensions were adjusted to 1 x 10^8 spores/ml.

Antimicrobial assay

The antimicrobial assay was performed by agar disc diffusion methods (Bauer et al., 1966: Nair et al., 2005). The Molten Muller Hinton agar (Himedia) was inoculated with 300 µl of the inoculum (1x 10^8 cfu/ml) and poured into the sterile Petri plates (Himedia).The disc (6 mm) was saturated with 50 µl of the extract (100 mg/ml) allowed to dry and was introduced on the upper layer of the seeded agar plate. The plates were incubated overnight at 35±2°C for 24 h. Microbial growth was determined by measuring the diameter of zone of inhibition. For each microbial strain, the solvent control treated as negative control. The results were obtained by measuring the zone of diameter. The experiment was done three times and the mean values are presented. The results were compared with the standard antibiotic streptomycin (0.5 mg/ml). Minimum inhibitory concentration (MIC), which was determined as the lowest concentration of plant extracts inhibiting the growth of the organism, was determined based on the readings.

RESULTS AND DISCUSSION

The phytochemical screening of leaf and *in vitro* calli extracts of *T. indica* revealed the presence of alkaloids, carbohydrates and steroids while using chloroform, ethanol and methanol (Table 1). Tannins and phenolic compounds were found only in the leaf extracts of chloroform, ethanol and water but they were absent in *in vitro* derived calli. The aqueous leaf and *in vitro* calli extracts were found to have saponin along with alkaloid and carbohydrates. It was observed that petroleum ether extracts of leaf and calli had only steroids as compared to other solvent extracts. Similarly, presence of alkaloids, carbohydrates, steroids, saponins, tannins and triterpenes was found in the alcoholic and aqueous root (Patel et al., 2006) and leaf (Gujrati et al., 2007) extracts of *T. indica*. It has been reported that these metabolites are of various pharmacological importance (Ndukwe et al., 2005) and possess antimicrobial properties (Cowan, 1999; Lewis and Ausubel, 2006).

The ethanobotanical screening tests of leaf extracts of *T. indica* in different solvents against gram positive and negative pathogenic bacteria and a fungal pathogen are depicted in Table 2. The minimum inhibiting concentration of the extracts to the tested bacteria was ranged from 20 to 80 mg/ml. In general most test organisms showed MIC of 20 mg/ml. The leaf extract in petroleum ether had 40 mg/ml against *S. aureus*, *B. subtilis* and *S. typhi*. Ethanol extract showed MIC of 60 mg/ml to *S. aureus* and 80 mg/ml to *P. vulgaris*. The MIC of 40 mg/ml against *S. faecalis*, *M. luteus*, *B. subtilis* and *P. vulgaris* but 60 mg/ml against *E. aerogenes* was obtained for the methanolic extract of leaf. The aqueous extract had high MIC of 50 to 80 mg/ml to the tested

Table 1. Phytochemical screening of leaf and callus extracts of *Tylophora indica.*

Solvent	Types of Extracts	Alkaloids	Carbohydrates	Saponins	Amino acids and protein	Fixed oils and fats	Gums and Mucilage	Phenolic compound	Tannins	Flavanoids	Steroids	Glycosides
Petroleum ether	Leaf	-	-	-	+	++	-	-	-	-	++	-
	Callus	-	-	-	+	-	-	-	-	-	++	-
Chloroform	Leaf	+++	+++	-	+	-	-	+	++	-	++	-
	Callus	+++	++	-	+	-	-	-	-	-	++	-
Ethanol	Leaf	+++	+++	-	+	-	-	+	++	-	++	-
	Callus	++	++	-	+	-	-	-	-	-	++	-
Methanol	Leaf	++	++	-	+	-	-	-	-	-	++	-
	Callus	++	++	-	+	-	-	-	-	-	++	-
Aqueous	Leaf	+++	+++	++	+	-	-	++	++	-	-	-
	Callus	++	++	+	+	-	-	-	-	-	-	-

+++, Highly present; ++, moderately present; +, present; -, absent.

Table 2. Minimum inhibitory concentration (MIC) of crude leaf and callus extracts from *Tylophora indica*

Test microorganism	Gram + / - or Fungi	MIC (mg/ml)										
		Petroleum ether		Chloroform		Ethanol		Methanol		Water		Streptomycin (mg/ml)
		Leaf	Callus	Leaf	Callus	Leaf	Callus	Leaf	Callus	Leaf	Callus	
Staphylococcus aureus	+	40.00	60.00	20.00	60.00	60.00	20.00	20.00	40.00	-	80.00	0.5
Streptococcus faecalis	+	20.00	-	20.00	-	20.00	-	40.00	-	80.00	-	0.6
Micrococcus luteus	+	-	80.00	-	80.00	20.00	60.00	40.00	60.00	80.00	80.00	0.5
Bacillus subtilis	+	40.00	40.00	20.00	40.00	20.00	60.00	40.00	20.00	60.00	80.00	0.3
Escherichia coli	-	20.00	40.00	20.00	20.00	20.00	20.00	20.00	20.00	60.00	40.00	0.5
Pseudomonasn aeruginosa	-	20.00	40.00	20.00	40.00	20.00	20.00	20.00	20.00	-	40.00	0.25
Klebsiella pneumoniae	-	20.00	40.00	20.00	40.00	20.00	20.00	20.00	40.00	80.00	40.00	0.3
Proteus vulgaris	-	20.00	-	20.00	-	80.00	-	40.00	-	-	-	0.15
Solmonella typhi	-	40.00	40.00	-	40.00	-	20.00	-	20.00	-	40.00	0.5
Enterobacter aerogenes	-	-	40.00	20.00	40.00	-	40.00	60.00	60.00	60.00	60.00	0.5
Candida albicans	Fungi	20.00	40.00	20.00	40.00	20.00	40.00	20.00	60.00	50.00	80.00	0.5

Values are the average of at least three determinations; -, no inhibition.

organisms compared to other solvent extracts. The differential MIC concentrations against pathogenic bacteria and fungi by solvent extracts of *Physalis minima* and *Rauvolfia tertraphylla* (Shariff et al., 2006), *Enatia chloratha* (Adesokan et al., 2008), *Marrubium valgare* (Masoodi et al.,

Table 3. The antimicrobial activity of leaf and callus extracts of *Tylophora indica.*

		Zone of inhibition (mm)										
Test Microorganisms	**Gram + / - or Fungi**	**Petroleum ether**		**Chloroform**		**Methanol**		**Ethanol**		**Water**		**Control (Strep. at 0.5 mg/ml)**
		Leaf	**Callus**	**Leaf**	**Callus**	**Leaf**	**Callus**	**Leaf**	**Callus**	**Leaf**	**Callus**	
Staphylococcus aureus	+	*	*	12.6±0.2	*	*	*	*	*	*	*	10.6±0.8
Streptococcus faecalis	+	13.8±0.9	8.2±0.8	16.0±2.5	*	12.4±1.1	*	12.2±0.4	11.0±1.0	9.6±1.1	8.4±0.5	11.6±1.1
Micrococcus luteus	+	*	*	*	*	8.2±0.8	8.2±0.8	12.2±0.4	9.8±1.0	9.2±1.5	8.4±0.5	7.0±0.7
Bacillus subtilis	+	14.2±1.4	8.2±0.8	11.8±1.3	8.0±0.1	9.8±0.8	9.8±0.8	*	*	12.0±0.8	*	18.6±0.9
Escherichia coli	-	13.0±1.2	7.8±0.8	15.4±0.5	9.8±0.8	14.4±2.1	11.8±1.3	13.0±1.2	11.2±1.0	14.6±1.1	*	20.4±1.1
Pseudomonas aeruginosa	-	*	*	*	*	13.0±0.7	*	12.8 ±0.8	12.0±1.0	*	14.0±0.7	14.2±0.8
Klebsiella pneumoniae	-	*	*	12.2±0.8	*	15.0±2.1	12.2±0.8	15.0± 0.7	14.6±1.5	16.0±0.7	17.0±0.7	11.2±0.8
Proteus vulgaris	-	*	7.8± 0.8	*	8.0±0.1	*	*	*	*	*	*	18.6±0.9
Solmonella typhi	-	14.2±1.9	8.4±0.8	*	*	15.0±0.7	11.2±0.8	*	21.0±1.0	14.6±0.8	*	10.0±0.7
Enterobacter aerogenes	-	*	7.8±1.1	*	6.8±0.8	*	9.8±0.8	9.2 ± 0.8	11.2±1.3	*	14.6±0.6	22.0±0.7
Candida albicans	Fungi	13.6±0.8	8.2±0.8	12.2±0.8	8.2±0.8	11.4±1.8	*	11.2±0.8	11.2±0.8	9.2±0.8	13.2±1.0	18.8±0.8

Values are the average of inhibition zone diameter of at least three determinations. *, No inhibition, Strep, antibiotic streptomycin. .

2008) and *Dichrotachys inerea* (Banso and Adeyemo, 2007) was also reported in earlier studies. It is not surprising that there are differences in the minimum inhibitory concentration of solvents extract due to the phytochemical properties. It is quite possible that some of the solvents extracts were effective at MIC levels and or ineffective implies qualitative and quantitative difference in antimicrobial constituents of the plant extracts and in addition to their intrinsic bioactivity by their ability to dissolve or diffuse in the *in vitro* assay media. Moreover the drying process may have caused conformational changes to occur in some of the chemical constituents found in plants (Parekh and Chanda, 2007).

The results for the antimicrobial activity tests (inhibition zone diameters (mm) for the extracts (100 mg/ml) and standard antimicrobial dose (Streptomycin at 0.5 mg/ml) against species of gram positive and negative bacteria and a fungus are given in Table 3. The solvents used for the dissolution of the leaf and calli extracts exhibited varying antimicrobial activity. Among the solvent extracts, petroleum ether extracts of leaf and calli had inhibitory action against four bacteria (*S. faecalis*, *B. subtilis*, *Esherichia coli*, *S.typhi*) but chloroform extracts found to inhibit two bacteria (*B. subtilis* and *E .coli*). Two Gram positive bacteria (*M. luteus*, *B.subtilis*) and three Gram negative bacteria (*E. coli*, *P. aeruginosa*, *S. typhi*) were sensitive to ethanolic extracts of leaf and calli .The methanolic extracts showed inhibitory action against two Gram positive bacteria (*S. faecalis* and *M. luteus*) and four Gram negative bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae* and *E. aerogenes*). Aqueous leaf extracts of both leaf and calli had inhibition against *S. aureus*, and *K. pneumoniae* alone. In most cases, solvent extracts of leaf had inhibitory activity against tested organisms better than extracts of calli. Moreover, the inhibitory activity of the extracts was not much significant except for thr ethanolic and methanolic extracts against *M. luteus, E. coli* and *S.typhi* as compared to standard antibiotic. The selected fungus *C. albicans* was found to be sensitive to all the extracts of leaf and calli. It was interesting to note that the calli extracts recorded sensitivity to *P. vulgaris*, *E. aerogenes*, *S. typhi* and *P. aeruginosa* with the chloroform, ethanol methanol and aqueous extracts, respectively. But it was not true with respective solvent extracts of leaf. Certain solvent extracts of leaf or calli were found ineffective against some Gram negative bacteria like *P. aeruginosa*, *K. pneumoniae*, *S. typhi and P. vulgaris.* It was also observed that the ethanolic and aqueous extracts of leaf and calli had inhibition against *M. luteus* but it was not inhibited by selected antibiotic in our study.

The different antimicrobial property of solvent

extracts of medicinal plants has been well reported by Parekh et al. (2005) and Nair et al. (2005). In our study, Gram positive bacteria were more susceptive to the solvent extracts than Gram negative bacteria. This might be due to the presence of outer membrane that appears as effective laminar in Gram negative bacteria (Nikaido, 1999). Moreover the solvent extracts of leaf and calli had inhibitory action against bacterial and fungal growth as reported by Shariff et al. (2006) but the efficacy of callus culture extracts was lower and potential against few selected pathogens. The results indicate the possibility of maintenance of the biological activity of plant in *in vitro* cultures. However, for optimization of appropriate culture conditions, careful selection of clones must be needed for production of pharmacologically important compounds (Manisha and Madumitha, 2000). It is hoped that this study would lead to establishment of antimicrobial compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin through *in vitro* culture methods besides focusing the minimization of using *in vivo* parts of the medicinal plants

REFERENCES

Adesokan AA, Yakubu MT, Owoyele BV, Akanji MA, Soladoye AO and Lawal, OK (2008). Effect of administration of aqueous and ethanolic extracts of *Enantia chlorantha* stem bark on brewer's yeast-induced pyresis in rats. Afr. J. Biochem. Res. 2(7):165-169.

Balandrin MF, Kjocket AJ, Wurtele E (1985). Natural plant chemicals; sources of industrial and mechanical materials. Science 228:1154-1160.

Banso A and Adeyemo SO (2007). Evaluation of antibacterial properties of tannis isolated from *Dichrostachys cinerea.* Afr. J. Biotechnol. 6(15):1785-1787.

Bauer AW, Kirby WMM, Sherris JC (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493-496.

Chandrasekar T, Mohamed Hussain T, Ramagopal G, Srinivasa Rao JV (2006). Somatic embryogenesis of *Tylophora indica* (Burm.f.) Merril, important medicinal plants. Int. J. Appl. Sci. Eng. 4(1):33-40.

Cowan MM (1999). Products as antimicrobial agents. Clin. Microb. Rev. 12:564-582.

Debnath M, Malik CP, Bisen PS (2006). Micropropagations: A tool for the production of high quality plant-based medicines. Curr. Pharm. Biotechnol. 7(1):33-49.

Gamble G (1935). Flora of Madras. Calcutta: Shree Saraswathi Press Ltd.

Gujrati V, Patel N, Rao VN, Nandakumar K, Gouda TS, Shanta SM (2007). Hepatoprotective activity of alcoholic and aqueous extracts of leaves of *Tylophora indica* (Linn.) in rats. Indian J. Pharmacol. 39:43-47.

Harborne LB (1973). Phytochemical methods. A guide to modern techniques of plant analysis. Chapman and Hall, London. p. 279.

Krishnaraju AV, Rao TVN, Sundaraju D (2005). Assessment of bioactivity of Indian medicinal plants using Brine shrimp (*Artemia salina*) lethality assay. Indian J. Appl. Sci. Eng. 2:125-134.

Lewis K, Ausubel FM (2006). Prospects for plant derived antibacterials. Nat. Biotechnol. 24(12):1504-1507.

Manisha T, Madhumita G. (2000). Biotechnology: A promising tool for conservation of phytodiversity. Plant Cell Biotechnol. Mol. Biol. 1(3 and 4):73-79.

Masoodi MH, Ahmed B, Zargar IM, Khan SA, Khan S, Singh P (2008). Antibacterial activity of whole plant extracts of *Marrubium vulgare.* Afr. J. Biotechnol 7(2):86-87.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15:473-497.

Nair R, Kalariya T, Chanda S (2005). Antibacterial activity of some selected Indian medicinal flora. Turk. J. Biol. 29:41-47.

Ndukwe G, Achimuga MO, Amako NF (2005). Phytochemical and antimicrobial screening of crude extracts from the stem bark of *Invingia gabonesis.* J. Pest Dis. Vector Mang. 6:391-397.

Nikaido H,(1999). Microdermatology; cell surface in the interaction of microbes with the external world. J. Bacteriol. 181:4-8.

Parekh J, Chanda S (2007). *In vitro* antimicrobial activity and phytochemical analysis of some Indian medicinal plants. Turk. J. Biol. 31:53-58.

Parekh J, Nair R, Chanda S (2005). Preliminary screening of some folklore medicinal plants from western India for potential antimicrobial activity. Indian J. Pharmacol. 37:408-409.

Patel NJ, Gujarati VB, Gouda TS Rao NV, Nandakumar K, Shantakumar SM (2006). Antidiarrhoeal activity of alcoholic and aqueous extracts of root of *Tylophora indica* (Wight and Arn.) in Rodents. Pharmacol. online 1:9-29.

Rastogi RP, Mehrotra BP (1998). Compendium of Indian Medicinal Plants, CDRI Lucknow and NISCOM, New Delhi. 5:868-870.

Shariff N, Sudarshana MS, Umesha S, Hariprasad P (2006). Antimicrobial activity of *Raulfia tertraphylla* and *Physalis minima* leaf and callus extracts. Afr. J. Biotechnol. 5(10):946-950.