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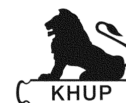

In vitro and *in vivo* antibacterial activities of root extract of tissue cultured *Pluchea indica* (L.) Less.

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SUMMARY

The methanolic root extract of tissue cultured *Pluchea indica* (L.) Less. was tested for its antibacterial potentiality against 102 different strains of bacteria belonging to both Gram positive and Gram negative groups. The bacteria could be arranged according to their decreasing order of sensitivity as follows: *Staphylococcus aureus*, *Vibrio cholerae*, *Bacillus* spp. *Vibrio parahaemolyticus*, shigellae, *Salmonella* spp., and *Escherichia coli*. The extract was found to be bacteriostatic in nature against *Salmonella typhimurium* NCTC 74. When administered to Swiss strain of white mice at the doses of 0.5 and 1.0 mg/kg body weight, the extract could significantly protect the animals challenged with 50 MLD of *S. typhimurium* NCTC 74. According to the chi-square test, the *in vivo* data is highly significant ($P < 0.001$).

Key words: Tissue cultured; *Pluchea indica*; Root; Antibacterial; *Salmonella typhimurium* NCTC 74

INTRODUCTION

One of the major achievements of medical science has been the control and management of infectious diseases. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. Thus plants should be thoroughly investigated to better understand their properties, safety and efficiency. Again plant tissue culture research is multi-dimensional and activities of tissue-cultured plants, if proven would no doubt revolutionize medicinal plant research. The main objective of this study is to evaluate the *in vitro* and *in vivo* antibacterial activities of tissue cultured *Pluchea indica* root extract against different bacterial virulent stains including *Salmonella typhimurium* NCTC 74.

The plant *Pluchea indica* (L.) Less. (Family: Asteraceae) is an evergreen large shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia and Australia. In Indo-China the roots in decoction prescribed in fevers as a diaphoretic and an infusion of the leaves is given internally in lumbago (Kirtikar and Basu, 1999). The root and leaves are used in some area as astringent and antipyretics (Kirtikar and Basu, 1999). The plant is also known to be used in rheumatoid arthritis (Chatterjee, 1996). Root extract has also been evaluated for (Sen *et al*., 1991), antiulcer (Sen *et al*., 1993), and neuropharmacological (Thongpraditchote

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et al., 1996) actions. The hypoglycemic and antihyperglycemic activity of leaf extract of *P. indica* (Pramanik *et al.*, 2006) and the antioxidant activity (Sen *et al.*, 2002) of roots of *P. indica* Less. have been already documented. The plant has also been reported to possess diuretic effects in traditional literature. 4 pure compounds isolated from its root have been reported to possess potent antimicrobial activities (Biswas *et al.*, 2005). So far a number of chemical constituents have been isolated from different parts of the plant. Previous phytochemical studies with *P. indica* revealed the occurrences of new eudesmane derivative from the roots (Mukhopadhyay *et al.*, 1983), five new terpenoid glycosides from aerial parts (Uchiyama *et al.*, 1989), three new eudesmane-type sesquiterpenes and three new lignan glycosides, together with a known eudesmane-type sesquiterpene from roots (Uchiyama *et al.*, 1991) and two new thiophene derivatives, besides two pentacyclic triterpenes from roots (Chakravarty *et al.*, 1994). A pure compound (R/J/3) isolated from the root of *P. indica* was found very potent against *E. histolytica* (Biswas *et al.*, 2006). Tissue cultured *P. indica* leave has also been reported to possess diuretic effect (Pramanik *et al.*, 2007).

MATERIALS AND METHODS

Tissue culture materials & methods (Pramanik *et al.*, 2007)

P. indica (L.) Less. plants were collected from the Diamond Harbour region of West Bengal, during their flowering stage and were identified by Dr. N. Paria (Department of Botany, University of Calcutta) and Sri Saibal Basu (Botanical Survey of India, Shibpur, India). The explants of authenticated *P. indica* (roots, stems with internodes) were excised, cleaned and were treated with 0.1% w/v Mercuric chloride solution followed by repeated rinsing with sterile distilled water to ensure no trace of the sterilant. The roots were then aseptically cut into 0.5 ± 0.2 sq. cm pieces and placed with their dorsal side on the agar. The shoots were cut into approximately 1 cm with 1 - 2 nodes possessing axillary buds and embedded erect in the medium. The MS (Murashige and Skoog, 1962) basal medium was supplemented with different concentrations of NAA (Naphthalene acetic acid), IAA (Indole acetic acid), 2,4-D (2,4-Dichlorophenoxyacetic acid), and BAP (6-Benzylamino-purine). All media were adjusted to pH 5.8 before autoclaving at 121 °C for 15 min. Sterilized explants (root, stems with internodes) were placed in MS media with various hormone combinations. Cultures were maintained properly. After 8 months, during flowering, the whole tissue-cultured plant was uprooted and the roots were separated, washed, treated with 1% sodium benzoate solution and dried at 55 °C up to 15% moisture content. It was then ground and extracted for evaluation of antimicrobial activities.

Material

The root of tissue cultured *P. indica* was separated, washed, oven dried at 60 °C, powdered and sieved through 100 meshes. Fibers and unwanted materials were rejected after sieving. The powder was preserved in an airtight container for further use.

Extraction

The pulverized root powder (500 g) of tissue cultured *P. indica* was extracted with methanol using a Soxhlet extractor to obtain the methanolic extract of *P. indica*. Then the solvent was evaporated under reduced pressure using a rotary evaporator (Model: H S - 2001 N) to obtain a semisolid residue. The yield of the extract was 8.7% w/w. The extract was suspended in 2% v/v aqueous tween 80 solution prior to the experiment.

Animals

Swiss strain of male white mice weighing 18 - 20 g was used for the *in vivo* studies. Animals were maintained at standard conditions at 21 ± 1 °C and 50 - 60% relative humidity with a photoperiod of 14 : 10 h of light-darkness. Water and a dry pellet diet were given *ad libitum*. The protocols applied in

Table 1. *In vitro* activity of *P. indica* root extract on Gram-positive and Gram-negative bacteria

Bacteria	No. of Bacterial strain tested	No. of strains inhibited by different concentrations of extract (μg/ml)					
		250	500	1000	1500	2000	>2000
Bacillus spp.	4		1	3	3	3	
Staphylococcus aureus	21	4	5	11	12	12	
Escherichia coli	17	2		4	7	8	
Salmonella spp.	6			2	3	3	
Salmonella typhimurium NCTC 74	1		1	1	1	1	
Shigella spp	18	3	7	14	14	14	
Vibrio cholerae	22	5	8	10	12	12	
Vibrio parahaemolyticus	13	3	6	8	13	13	
Total	102	17	28	53	65	66	

this study have been approved by the Departmental Animal Ethics Committee.

Bacterial strains

The strains used in this study were *Staphylococcus aureus*, *Bacillus spp.*, *Shigella boydii, Salmonella typhimurium, Escherichia coli* and *Vibrio cholerae*. All these were received from National Collection of Type Culture (NCTC), London or American Type Culture Collection, USA (Table 1). These strains were identified as described by Collee *et al*. (1996) and preserved in freeze-dried state.

Media

Liquid media used for this study were peptone water [PW, Oxoid brand bacteriological peptone 1% (w/v) plus Analar NaCl 0.5% (w/v)], nutrient broth (NB, Oxoid) and Mueller Hinton broth (MHB; Oxoid). Solid media were peptone agar (PA), nutrient agar (NA) and Mueller Hinton agar (MHA), obtained by solidifying the respective liquid media with 1.2% (w/v) agar (Oxoid No.3); another solid medium used was desoxycholate citrate agar (DCA, Oxoid). The pH was maintained at 7.2 - 7.4 for all the media. NA was used for tests with Gram-positive bacteria and PA and DCA were used for the rest of the bacteria as needed.

Determination of minimum inhibitory concentration (MIC)

The extract was added at concentrations of 0 (control), 500, 1000, 1500, and 2000 μg/ml molten NA and poured in Petridishes according to National Committee for Clinical Laboratory Standards. The organisms were grown in NB or PW for 18 h and harvested during the stationary growth phase. A direct suspension of the organisms was prepared in 5 ml sterile distilled water. The turbidity of the suspension was adjusted to match a 0.5 McFarland's standard (McFarland *et al.*, 1907) with a spectrop-hotometer (Chemito UV 2600 Double Beam UV-Vis Spectrophotometer) at 625 nm, which corresponded to 2.4×10^8 colony forming units (cfu)/ml. The inocula were prepared by further diluting the suspension 1:100 with sterile distilled water so that a 2 mm diameter loopful of a culture contained 10^5 cfu. These were spot-inoculated on the NA plates containing increasing amounts of the drug, including a control. The plates were incubated at 37 °C, examined after 24 h and incubated further for 72 h, where necessary. The lowest concentration of the drug in a plate that failed to show any visible macroscopic growth was considered as its MIC. The MIC (MIC 50 and MIC 90) determination was performed in triplicate for each organism and the experiment was repeated where necessary.

Mechanism of antibacterial action

The MIC of the root of tissue cultured *P. indica* against *S. typhimurium* NCTC 74 was found to be 500 μg/ml. At the logarithmic growth phase of the culture, the cfu counts of the strains were taken, and twice the MIC of the extract (500 μg/ml) was added to the culture. Subsequently, the cfu counts of the cultures were determined after 2, 4, 6 and 18 h of adding the drug.

***In vivo* tests**

The virulence of the test strain *S. typhimurium* NCTC 74 was exalted by repeated mouse passage and the median lethal dose (MLD or LD_{50}) of the passaged strain corresponding to 1.85×10^9 cfu/mouse suspended in 0.5 ml nutrient broth served as the challenge dose for all the groups of animals. Reproducibility of the challenged dose was ensured by standardization of its optical density in a Klett-Summerson colorimeter at 640 nm and determination of the cfu count in NA.

To determine the toxicity of the extract, 40 mice were taken, 20 of which was injected with 1 mg/Kg body weight of the extract, and the rest 20 received 0.5 mg/kg body weight of the same. They were kept under observation upto 100 h.

Two groups of mice, 20 animals per group (each mouse weighing 20 g) were kept in separate cages. Group I was intraperitoneally administered with 0.5 mg/kg body weight of the extract per mouse, and group II was given 1mg/Kg body weight of the extract per mouse. After 3 h, each group I and II was challenged with 50 MLD of *S. typhimurium* NCTC 74. A control group of 60 mice was also injected similarly with the same bacterial strain, and 0.1 ml sterile saline instead of the test extract. The protective capacity of the plant extract was determined by recording the mortality of the mice in different groups up to 100 h of the treatment, and statistically done by χ^2 test.

In another experiment, 4 groups of mice, 5 animals per group, were taken. Groups I and III were administered with 1 mg/kg body weight of the extract, while groups II and IV were given 0.1ml sterile saline. After 3 h, all the groups were given a 50 MLD challenge of *S. typhimurium* NCTC 74. After 2 h, groups I and II were sacrificed. Their heart blood was collected aseptically; their livers and spleens were removed aseptically and homogenised in tissue homogenisers. Cfu counts of the individual organs were determined separately. The same procedure was applied on groups III and IV at 18 h after the challenge. Statistical analysis of the *in vivo* data was done by Student's *t*-test.

RESULTS

Tissue cultured *P. indica* root extract was screened against 102 strains of bacteria (Table 1). Out of 4 strains of *Bacillus* spp., 1 were inhibited by the extract within the concentration of 500 μg/ml; among 21 strains of *Staphylococci* tested, 4 were inhibited at 250 μg/ml, 5 were inhibited at 500 μg/ml, 11 at 1000 μg/ml, and 12 at 1500 μg/ml. With respect to strains of *Salmonella* spp., all the 6 strains tested were inhibited at 1000 - 2000 μg/ml of the extract. *S. typhimurium* NCTC 74 stopped growing at 500 μg/ml of the drug. For *Shigella*, MIC ranged from 250 - 1000 μg/ml for 14 strains out of 18 tested. Among 22 strains of *V. cholerae*, 5 could not grow at 250 μg/ml of the extract, 12 strains were inhibited at 500 - 1500 μg/ml. All the strains

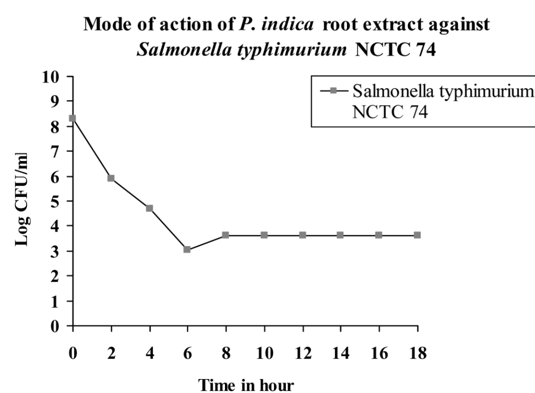

Fig. 1. Mode of action of *P. indica* root extract against *S. typhimurium* NCTC 74.

of *V. parahaemolyticus* got inhibited with 1500 µg/ml of the test extract. The action of the extract was less significant against *E. coli*.

For bacteriostatic activity of methanolic extract of *P. indica* extract at the logarithmic growth phase of the culture of *S. aureus* NCTC 6571, the cfu count of the strain was 2×10^8. Subsequently, the cfu of the culture was 8.0×10^5 after 2 h, 5.2×10^4 after 4 h, 11.0×10^2 after 6 h and 4.0×10^2 at the end of 18 h (Fig. 1). Similar bacteriostatic action was recorded in *V. cholerae* 1347.

Table 2 shows that in the control group, 49 out of 60 animals died within 100 h of the challenge and no mortality was recorded in those groups of mice that received different doses of the extract only. There was a significant protection in the extract-treated groups. In Table 3, it is seen that the extract significantly reduced the number of viable bacteria

Table 2. Determination of *in vivo* protective capacity of methanolic extract of root of *P. indica in vivo*

Control group* Drug injected per mouse	Mice died (out of 60)	Test group* mg/kg body weight	Mice died (out of 20)
0.1 ml sterile saline	49	0.5	16
		1	0

*Received a challenge dose of 1.85×10^9 cfu in 0.5 ml NB of *S. typhimurium* NCTC 74. None of the animals died when 0.5 and 1mg/Kg body weight of the extract was injected to 2 separate groups of mice (20 mice in each), i.e., the extract was found to be totally non-toxic to mice. $P < 0.001$, according to Chi-square test.

Table 3. Reduction in CFU/ml of *S. typhimurium* NCTC 74 in organ homogenates of mice treated with *P. indica* extract

Time of sampling	Group	Mouse No.	Drug/Kg body weight	Cfu/ml counts in		
				Heart blood	Liver	Spleen
2 h	I	1	Extract 1 mg	2.1×10^3	1.1×10^3	4.3×10^3
		2		2.3×10^3	3.0×10^3	4.6×10^3
		3		2.5×10^3	6.5×10^4	1.2×10^3
		4		3.1×10^4	2.1×10^3	6.2×10^3
		5		5.6×10^3	1.2×10^4	2.5×10^4
2 h	II	1	Saline (Control)	5.7×10^6	2.8×10^6	8.4×10^6
		2		4.0×10^5	$4,6 \times 10^6$	1.2×10^5
		3		5.8×10^5	6.0×10^6	5.4×10^6
		4		6.9×10^6	7.0×10^6	8.6×10^5
		5		7.8×10^6	8.5×10^6	8.8×10^6
18 h	III	1	Extract 1mg	3.6×10^2	5.8×10^2	7.8×10^2
		2		2.6×10^2	7.3×10^2	3.5×10^2
		3		4.5×10^2	3.8×10^2	7.2×10^3
		4		1.1×10^3	2.3×10^2	4.0×10^2
		5		7.0×10^2	7.1×10^2	3.4×10^2
18 h	IV	1	Saline (Control)	4.7×10^8	5.8×10^8	5.0×10^8
		2		5.4×10^8	5.2×10^8	5.4×10^9
		3		6.8×10^7	2.7×10^7	8.2×10^8
		4		5.6×10^8	3.9×10^7	4.9×10^7
		5		7.2×10^9	8.0×10^8	1.8×10^7

Viable counts between two groups significant; $P < 0.05$ in 2 h samples and $P < 0.001$ in 18 h samples.

in heart blood, liver and spleen of mice, both at 2 h and 18 h after challenge when compared with the control (saline treated) mice. Statistical analysis showed $P < 0.05$ for 2 h samples and $P < 0.001$ for 18 h samples.

DISCUSSION

Methanolic root extract of tissue cultured *P. indica* was found to possess antibacterial activities. While sensitive bacterial strains occurred among *Staphylococcus aureus Bacillus* spp., *Vibrio* spp., and some *shigellae*, the drug was much less active with respect to strains of *E. coli*. It may be pointed out here that the drug revealed pronounced inhibitory action against *Salmonella* spp. an organism, which is known to be the causative agent of typhoid fever. The extract was found to be bacteriostatic when tested *in vitro* against Gram-negative bacteria. The animal experiments were undertaken to determine its relevance to human therapeutic application. The results of *in vivo* tests were significant.

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