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In vitro and *In vivo* Antibacterial Activities of root extract of tissue cultured *Pluchea indica* (L.) Less. against bacillary dysentery.

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ABSTRACT

The methanolic root extract of tissue cultured *Pluchea indica* (L.) Less was tested for its antibacterial potentiality against 18 strains of *Shigella* species which is well known gram-negative bacillus functioning as common pathogen in humans. In the *in vitro* MIC study total 18 shigella strains were tested, among them *Sh. boydii* 10, *Sh. dysenteriae* 1, *Sh. dysenteriae* 7 and *Sh. flexneri* 2a NK 307 were inhibited at 250 µg/ml. Again *Sh. boydii* 8, *Sh. boydii* 10, *Sh. boydii* B 22461, *Sh. dysenteriae* 1, *Sh. dysenteriae* 2, *Sh. dysenteriae* 3, *Sh. dysenteriae* 6, *Sh. flexneri* 5a 18603 were inhibited at a concentration of 500 µg/ml, while the stain like *Sh. boydii* D 13624, *Sh. dysenteriae* 8 were inhibited at a concentration of 1000 µg/ml. The extract was found to be bacteriostatic in nature against *Sh. flexneri* 2a NK 307. In the *in vivo* experiment using the rabbit ileal loop model two different dosages of *P. indica* root extract (500 µg/ml and 1000µg/ml) were able to protect the animals when they were challenged with *Sh. flexneri* 2a NK 307 in the ileum. The results obtained suggest marked antibacterial activity of the root extract of tissue cultured *Pluchea indica*.

KEYWORDS: Antibacterial, *Sh. flexneri* 2a NK 307, tissue Cultured, *Pluchea indica* root.

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 proved would no doubt revolutionize medicinal plant research. The main objective of this study has been to evaluate the *in vitro* and *in vivo* antibacterial activity of tissue cultured *Pluchea indica* root extract against virulent stains *Shigella flexenerii* 2a NK 307 by rabbit ileal method.

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 are prescribed in fevers as a diaphoretic and an infusion of the leaves is given internally in lumbago (1). The root and leaves are used in some place as astringent and antipyretics (1). The plant is also known to be used in rheumatoid arthritis (2). The root extract has also been evaluated to possess anti-inflammatory (3), antiulcer (4), and neuropharmacological actions (5). The hypoglycemic and antihyperglycemic effect of leaf (6) and antioxidant activity (7) of roots of *P. indica* Less. are already reported. Four of the pure compounds isolated from its root have been reported to possess potent antimicrobial activities (8). So far a number of chemical constituents have been isolated from different parts of the plant. A new eudesmane derivative from the leaves (9), five new terpenic glycosides from aerial parts (10), three new eudesmane-type sesquiterpenes and three new lignan glycosides, together with a known eudesmane-type sesquiterpene from roots (11) and two new thiophene derivatives, besides two pentacyclic triterpenes of rare occurrence from roots (12) have been isolated from this plant. A pure compound (R/J/3) isolated from the root of *P. indica* was found very effective against *E. histolytica* (13). Tissue culture of the plant *P. indica* is done for getting more secondary metabolites. Tissue cultured *P. indica* leave is also reported to possess

diuretic effect (14).

MATERIALS AND METHODS

Tissue culture (14)

Pluchea 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 *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 (15) 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-Benzylaminopurine). All media were adjusted to pH 5.8 before autoclaving at 121°C for 15 minutes. Sterilized explants were placed in media with various hormone combinations. Cultures were maintained in tubes of 10 ml medium and kept at a temperature of 22°C ± 2°C with a relative humidity of 78% under 18 hours photoperiod (140 – 180 mmol/m^2/s). After 30 days of culture, the callus and regenerated shoots were transferred to fresh media. The procedure was repeated to obtain their potential for root regeneration.

The regenerated plantlets, after careful separation, were transferred to 1:1 cocopit: rice-husk mixture and gradually exposed for acclimatization to normal room temperature. After two weeks the hardened plants were potted in the garden soil (Photograph 1).

After 8 months, during flowering, the whole 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 *Pluchea indica* were 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 powder (500 gm) was extracted with methanol using a Soxhlet extractor to obtain the methanolic extract of *P. indica* (MEPI). Then the solvent was evaporated under reduced pressure using a rotary evaporator 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 and used.

Animal

New Zealand white rabbit, healthy male of 2kg body weight was used for the *in vivo* study. The animals were kept under standard conditions at 21± 1°C and 50-60% relative humidity with a photoperiod of 14:10h of light darkness. The rabbit was fasted for 24 hours before the experiment but water was provided *ad libitum*.

Bacteria

A total of 18 strains of *Shigella* were tested *in vitro* of which 18 are of human origin, identified as described by Barrow and Feltham (16). *Shigella flexneri* 2a is of rabbit origin. All are 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 MIC of the methanolic root extract of tissue cultured *P. indica* was accurately determined by spotting (in triplicate) 10^5 colony-forming unit (CFU) on nutrient agar plates containing the extract at the following concentration (µg/ml): 0 (control), 250, 500, 1000, 1500, 2000 and 2500 µg/ml molten NA and poured in Petridishes according to NCCLS (17). 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 (18) with a spectrophotometer (Chemito UV 2600 Double Beam UV-Vis Spectrophotometer) at 625 nm, which corresponded to 2.4 x 10^8 colony forming units (CFU)/ml. The inocula were prepared by further diluting the suspension 1:100 with sterile distilled

water such 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 (Table 1). The MIC (MIC 50 and MIC 90) determination was performed in triplicate for each organism and the experiment was repeated where necessary.

Determination of mode of action

Shigella strain sensitive to MEPI was chosen, namely *Sh. flexneri* 2a NK 307. Bacterial strain was grown in 4 ml of nutrient broth for 18 hours. Then, 2 ml of this culture was added to 4 ml of fresh nutrient broth and incubated at 37° C for 2 hours to help the strain to attain logarithmic growth phase. At this stage, the CFU count was determined and MEPI was added at a concentration higher than the respective minimum inhibitory concentration (MIC). CFU counts from the cultures were individually taken after 2, 4, 6 and 18 h of adding the drug.

The MIC of MEPI against *Sh. Flexneri* 2a NK 307 was found to be 250 μg/ml. At the logarithmic growth phase of the cultures, when the CFU counts of the strain was 1.02×10^9, 500 μg/ml (double the MIC) of the extract was added to the culture. Subsequently, the CFU of the culture was determined. CFUs were 1.04×10^8 after 2 h, 1.35×10^6 after 4 h, 2.24×10^4 after 6h and 2.24×10^3 at the end of 18 h (Fig.1) The result revealed the bacteriostatic nature of the test tissue cultured *P. indica* extract.

In vivo tests

In the *in vitro* study the effect of tissue cultured *P. indica* extract shows significant antimicrobial activity against *Shigella flexenerii* 2a NK 307. Rabbits were anesthetized with proper anaesthetic and the intestines were exteriorized through a midline incision by aseptic technique. Loops were prepared in the ileum by suturing the intestine at 3-5 cm intervals and separating loops with 1.5 to 2.5cm inter loops.

Strain of *Sh. flexneri* 2a NK 307 (a rabbit virulent strain) was grown initially in solid nutrient agar medium. From the pure culture one loopful of culture was transferred into 5 ml peptone water to prepare 24 hours old culture and the number of cells was adjusted to approximately 10^9 CFU / ml. Four loops were prepared in the externalized rabbit ileum using proper ligation. Starting with the negative control loop followed by lower concentration of extract-administered loop, higher concentration of extract administered loop and positive control loop were prepared accordingly. Except negative control loop all other loops were administered with 24 hours old culture of *Sh. flexneri* 2a NK 307. Negative control loop was administered with 0.7 ml of fresh peptone water only to make up the volume of the loop so that each loop shows the same swelling at the onset since negative control loop was not given the test compound extract. The next loop after negative control was administered with 0.5 ml of culture and 500 μg / ml of extract (0.2 ml from a stock solution of 2.5 mg / ml of extract). Then the third loop was given 0.5 ml of culture of *Sh. flexneri* 2a NK 307 and 1000 μg / ml of extract (0.2 ml from a stock solution of 5 mg / ml of extract). Fourth loop or the positive control loop was then given only 0.7 ml of culture of *Sh. flexneri* 2a NK 307. Finally the ligated rabbit ileum was again packed inside the body and the skin layers were sutured perfectly. Then Rabbit was taken back in the cage. After 18 h, the rabbit was sacrificed using chloroform (E. Merck). Fluid was collected separately and aseptically from each loop of the ileum. After several dilutions CFU count of individual loop was determined aseptically (Table 2).

RESULTS

In the *in vitro* MIC study total 18 *Shigella* stain were tested (Table-1). Among them *Sh. boydii* 10, *Sh. dysenteriae* 1, *Sh. dysenteriae* 7and *Sh. flexneri* 2a NK 307 were inhibited at 250 μg/ml.again *Sh. boydii* 8, *Sh. boydii* 10, *Sh. boydii* B 22461, *Sh. dysenteriae* 1, *Sh. dysenteriae* 2, *Sh. dysenteriae* 3, *Sh. dysenteriae* 6, *Sh. flexneri* 5a 18603 were inhibited at a concentration of 500 μg/ml, while the stain like *Sh. boydii* D 13624, *Sh. dysenteriae* 8 were inhibited at a concentration of 1000 μg/ml and *Sh. boydii* BCH 937, *Sh. dysenteriae* 5 were inhibited at a concentration of 1500 μg/ml and only one stain *Sh. flexneri* 2a 33220 is inhibited at 2000 μg/ml. The remaining 1 strain, *Sh. flexneri* 3a 30903 was found to be inhibited at much higher concentration by the extract (2500 μg/ml) and was considered to be resistant.

Thus it is evident from the text that the strains inhibited at 250 μg/ml are said to be highly sensitive towards the compound. Strains which got inhibited at 500 μg/ml, 1000 μg/ml and 1500 μg/ml can be considered as moderately sensitive and rest of the strains inhibited at 2000 μg/ml can be less sensitive

Table 1. In vitro activity of P. indica root extract on Shigella spp.

Bacterial Strains tested	Growth in nutrient agar plate containing different concentrations of *P. indica* (µg/ml)						
	0*	250	500	1000	1500	2000	2500
Sh. boydii 8	+	+	-	-	-	-	-
Sh. boydii 10	+	-	-	-	-	-	-
Sh. boydii BCH 937	+	+	+	+	-	-	-
Sh. boydii B 22461	+	+	-	-	-	-	-
Sh. boydii D 13624	+	+	+	-	-	-	-
Sh. boydii E 16552	+	+	+	-	-	-	-
Sh. dysenteriae 1	+	-	-	-	-	-	-
Sh. dysenteriae 2	+	+	-	-	-	-	-
Sh. dysenteriae 3	+	+	-	-	-	-	-
Sh. dysenteriae 5	+	+	+	+	-	-	-
Sh. dysenteriae 6	+	+	-	-	-	-	-
Sh. dysenteriae 7	+	-	-	-	-	-	-
Sh. dysenteriae 8	+	+	+	-	-	-	-
Sh. flexneri 2a NK 307	+	-	-	-	-	-	-
Sh. flexneri 3a 30903	+	+	+	+	+	+	-
Sh. flexneri 4a 24	+	+	+	-	-	-	-
Sh. flexneri 2a 33220	+	+	+	+	+	-	-
Sh. flexneri 5a 18603	+	+	-	-	-	-	-

**, Control plate without the drug; +, Growth; –, No growth.*

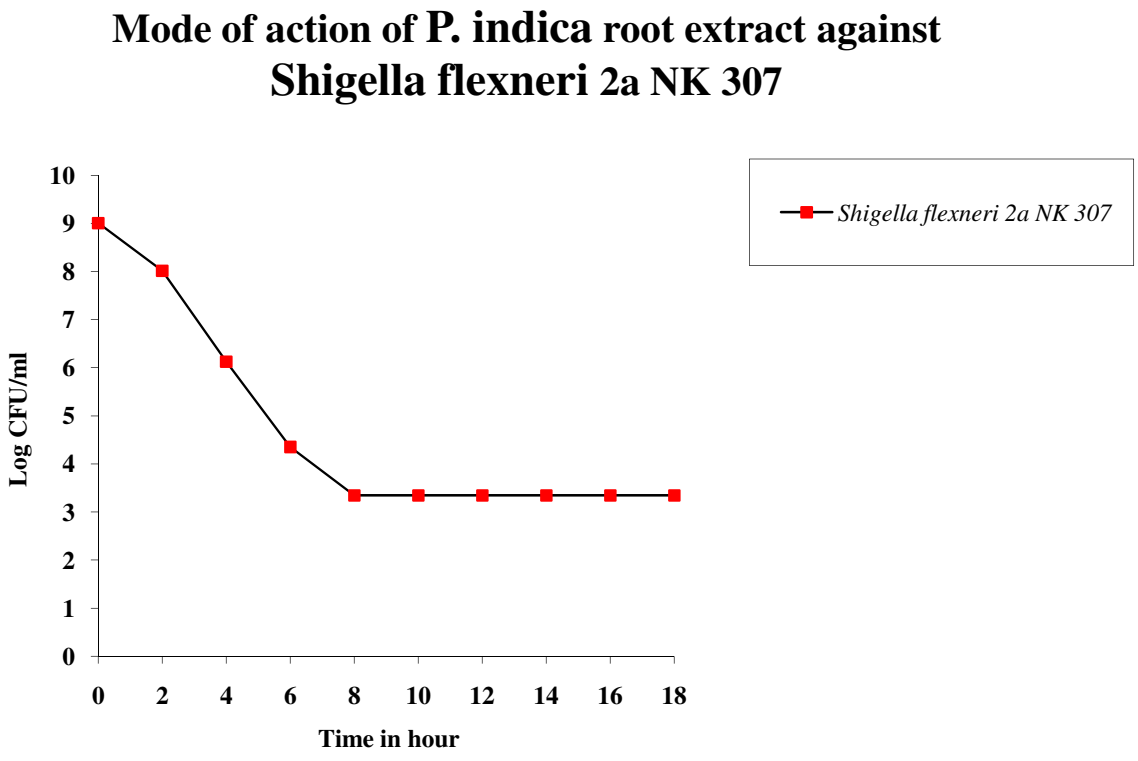


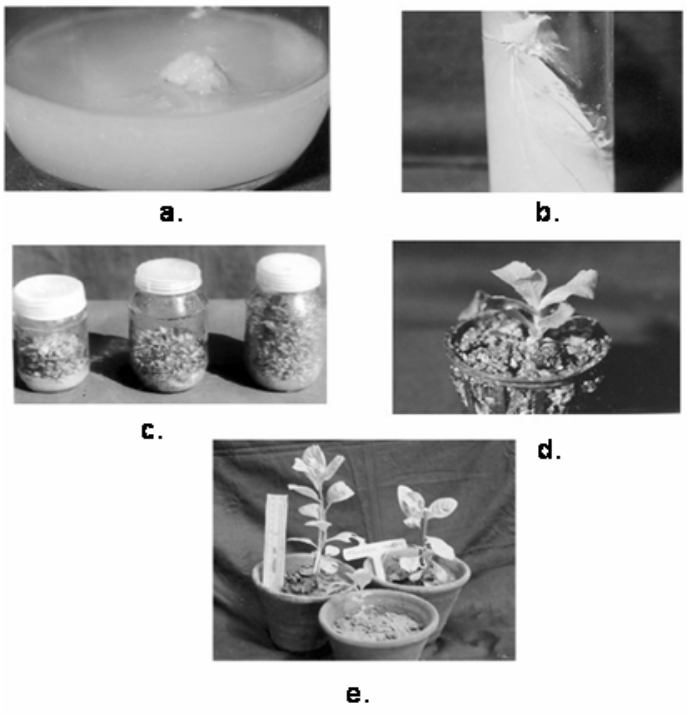
Fig. 1. Mode of action of P. indica root extract against Shigella flexenerri 2a NK 307.

Table 2. In vivo activity of methanolic root extract of P. indica on Sh. flexneri 2a NK 307 in different loops of rabbit ileum.

Type of Experiment	Count of CFU / ml
Negative control loop*	0
Loop containing lower dose of MEPI (500µg / ml)	2.35×10^6
Loop containing higher dose of MEPI (1000µg / ml)	4.1×10^3
Positive control loop**	2.1×10^9

* Received only peptone water ; ** Received only the culture of *Sh. flexneri 2a* NK 307.

Photograph 1. Different stages of Tissue culture of P. indica



a. Callus initiation from leaf explants cultured in MS + 0.875 µM IAA after 2 weeks.
b. Direct root generation from root explants cultured in MS+ 0.75 µM IAA after 3 weeks.
c. Comparative growth of P. indica shoots in Medium I, II, III.
d. Plantlets established in Vermiculite.
e. Potted Tissue cultured plants of P. indica in garden soil.

and 2500 µg/ml can be regarded as resistant.

In vivo activity was determined by administering rabbit virulent strain namely *Sh. flexneri* 2a NK 307 into the ileal loops of a male New Zealand white rabbit. Of the four loops prepared, the negative control loop, as it contains no culture, showed absence of bacterial growth and the positive control loop, as it contains the highest volume of bacterial culture, exhibited the presence of highest number of CFU/ml. In comparison to that other two loops containing two different concentrations of the compound (500 µg/ml and 1000 µg/ml respectively) of the said compound showed reduced number of CFU/ml. 1000 µg/ml concentration of MEPI showed significant reduction in the bacterial count (Table 2).

DISCUSSION

Shigella species are commonly pathogenic to humans, causing severe gastroenteritis (bacillary dysentery). In healthy adults, dysentery is a self-limiting disease, but it can be fatal to infants and young children, causing over 1 million deaths a year. In developing countries, the most common *Shigella* species are *S. flexneri* (mainly serotypes 2a, 1b, 3a, 4a and 6) and *S. sonnei*. After oral inoculation, *shigellae* pass to the terminal

ileum and colon where they invade and proliferate within epithelial cells, spreading from cell to cell. Bacillary dysentery is an invasive infection of the colonic and rectal mucosa that tends to remain local. In severe cases, the invasive process also may affect the terminal ileum. The disease process is characterized by an acute inflammation of the intestinal mucosa with ulcerations of the epithelium. The pathogenesis of *Shigella* can be studied in various animal models that variably reproduce the invasive and pro-inflammatory capacity of the microorganism. The almost exclusive specificity of *Shigella* for humans explains the lack of an animal model that mimics the natural disease. Nevertheless, several model systems manifest different facets of *Shigella* enteropathogenicity. The rabbit ligated intestinal (ileal) loop model, which manifests the rupture of the intestinal barrier, invasion and tissue destruction, and also allows study of the role of the follicle-associated epithelium that covers mucosa-associated lymph nodes in epithelial translocation of *Shigella* (19, 20).

Methanolic root extract of *P. indica* was found to possess antibacterial activity both *in vitro* and *in vivo* against *Sh. flexneri* 2a NK 307 was able to show good antidysentric activity while tested against a number of *Sh. flexneri* strains. An analysis of the result in Table1 shows that out of 18 strains of *Shigella* spp., the MIC of the extract was 250 µg/ml with respect to 3 strains, 500 µg/ml against 7 strains, 1000 µg/ml with respect to 4 strains, 1500 µg/ml against 2 strains and 2000 µg/ml with respect to 1 strain. The remaining 1 strain was found to be inhibited at much higher concentration by the extract (2500 µg/ml) and was considered to be resistant.

The MIC of MEPI against *Sh. Flexneri* 2a NK 307 was found to be 250 µg/mL. At the logarithmic growth phase of the cultures, when the CFU counts of the strain was 1.02×10^9, 500 µg/ml (double the MIC) of the extract was added to the culture. Subsequently, the CFU of the culture was determined. CFUs were 1.04×10^8 after 2 h, 1.35×10^6 after 4 h, 2.24×10^4 after 6h and 2.24×10^3 at the end of 18 h. The result revealed the bacteriostatic nature of the test tissue cultured *P. indica* extract.

More over the compound was totally non-toxic to animals even at very high concentrations. The *in vivo* experiment was also performed in rabbit ileum in order to establish the antidysentric activity of the tissue cultured *P. indica* extract. The protection rendered by the compound to rabbit ileum infected with *Sh. flexneri* 2a NK 307 was found to be statistically highly significant. The extract was found to be bacteriostatic when tested *in vitro* against a Gram-negative bacteria. The results of *in vivo* tests were significant. The animal experiments were undertaken to determine its relevance to human therapeutic application. Again this finding has corroborated our earlier studies to establish medicinal plants and phytochemicals as an outstanding alternative. Furthermore, synergistic combination with other phytochemicals may improve the therapeutic index.

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