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Tissue culture of the plant *Pluchea indica* (L.) Less. and evaluation of diuretic potential of its leaves

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SUMMARY

The present study focused on the establishment of micropropagation protocol for the high value *Pluchea (P.) indica* (L.) Less., genotype, an important medicinal plant and evaluation of the diuretic activity of the leaf extract of the tissue cultured plant. Leaf explants, nodal segments and shoot tips were cultured in MS medium supplemented with auxin and cytokinin and their combinations. With the objective of inducing callus giving rise to new adult plants, naphthalene acetic acid was found to be most effective for (80%) for callus induction. The methanolic extract of leaves of the micropropagated *P. indica* was investigated for its diuretic activity in Wistar albino rats. Urinary excretion parameters were studied for evaluation of diuretic activity using Frusemide (20 mg/kg, p.o.) as standard. The extract showed significant diuretic activity at the doses of 100, 200 and 300 mg/kg. p.o. An oral acute toxicity study for the extract was carried out and the LD_{50} value was found to be 2,825 mg/kg body weight.

Key words: *Pluchea indica*; Micropropagation; Leaves; MS medium; Diuretic

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 grow the plant *Pluchea (P.) indica* by exploiting the tissue culture techniques and thereby evaluate its diuretic activity. For ease of understanding, the tissue culture and diuretic studies have been treated separately.

The plant *P. 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. The roots and leaves are reported to possess astringent and antipyretic properties and are given in decoction as a diaphoretic in fevers. 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. The root and leaves are used in Patna as astringent and antipyretics (Kirtikar and Basu, 1999). The plant is also known to be used in rheumatoid

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arthritis (Chatterjee, 1996). The root extract has also been evaluated to possess anti-inflammatory (Sen *et al.*, 1991), antiulcer (Sen *et al.*, 1993), and neuropharmacological (Thongpraditchote *et al.*, 1996) actions. The hypoglycemic effect (Peungvicha *et al.*, 1999) and antioxidant activity (Sen *et al.*, 2002) of roots of *P. indica* Less. are already reported. The plant has also been reported to possess diuretic effects in traditional literature. Four of the 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. A new eudesmane derivative from the leaves (Mukhopadhyay *et al.*, 1983), five new terpenic 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 of rare occurrence from roots (Chakravarty *et al.*, 1994) have been isolated from this plant.

MATERIALS AND METHODS

Collection and authentication of the plant material

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).

Preparation of explants

The explants (leaves, 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 leaves 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.

Material

The leaves of tissue cultured *P. 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 g) 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 7.9% w/w. The extract was suspended in 2% v/v aqueous Tween 80 solution prior to the experiment and used.

Chemicals and drugs used

All the chemicals used were obtained from S.D. Fine Chemicals Limited, Mumbai, India. Frusemide (Recon, Bangalore, India) was used as standard drug.

Animals

Wistar albino rats of either sex, weighing 150 - 200 g were used. The animals were kept under standard conditions of 12:12 h light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *ad libitum*. The entire study was approved by the institute animal ethical committee and carried out in an ethically proper way by following guidelines as set by the World Health Organization (WHO, 2000).

Toxicity studies

Acute toxicity relating to the determination of LD_{50} value was performed with different doses of the extract (Ghosh, 1994) that revealed that the extract is safe to use even at the doses of 2,825 g/kg of body weight orally.

Evaluation of diuretic activity

The method of (Lipschitz *et al.*, 1943) was followed for the evaluation of diuretic activity. Rats were divided into six groups of six animals each. Group I served as untreated control and Group II received 2% v/v aqueous Tween 80 solution (10 ml/kg p.o.); Group III received Frusemide (20 mg/kg p.o.) as standard; Groups IV, V and VI received MEPI at doses of 100, 200 and 300 mg/kg p.o., respectively. Twenty four hours prior to the experiment, the test animals were placed into metabolic cages with total withdrawal of food and water. After oral administration of test samples, the urinary output of each group was recorded over the span of 8 h. Diuretic activity was observed at a temperature of 25 ± 0.5°C. The concentration of Na^+ and K^+ were analyzed by flame photometry, and the amount of Cl^- was determined by Argentometric titration method (Ramesh *et al.*, 1996).

Maintenance of cultures

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 ± 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.

Hardening of plantlets

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

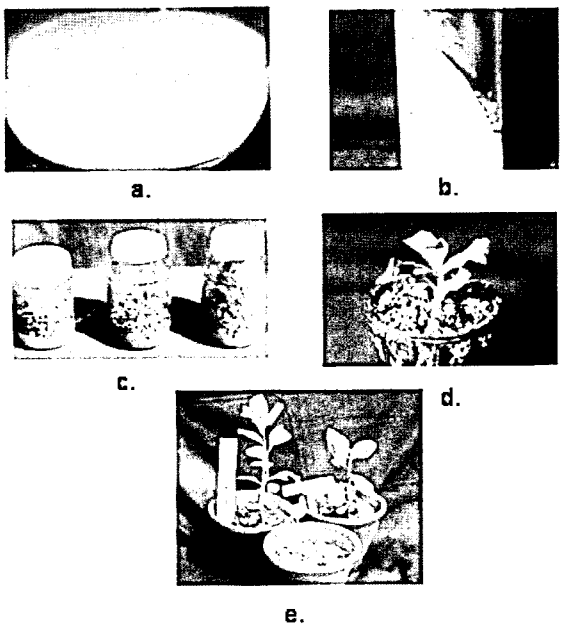


Fig. 1. Photographs of Pluchea. **a.** Callus initiation from leaf explants cultured in MS + 0.875 μM IAA after 2 weeks (× 1). **b.** Direct root generation from root explants cultured in MS + 0.75 μM IAA after 3 weeks (× 1.5). **c.** Comparative growth of *P. indica* shoots in Medium I, II, III (× 0.25). **d.** Plantlets established in Vermiculite (× 0.1). **e** Potted tissue cultured plants of *P. indica* in garden soil (× 0.1).

Data analysis

All data were recorded at 30 days interval.

Statistical analysis

The results were expressed as mean ± SEM. The data were analyzed for significance using the one-way ANOVA followed by Dunnett's test. *P* values less than 0.05 were considered as significant.

RESULTS AND DISCUSSION

Callus induction

Wide ranges of calli types were obtained when the leaf explants were cultured on MS basal medium supplemented with different hormone concentrations, the site of callus induction varying with the type of hormone used. Slight variation in frequency of response was observed among the leaf explants

when treated with different concentrations of the three growth regulators. In all cases 0.5 ml/l hormone concentration showed the best result with maximum frequency. Among the three hormones, the concentrations of IAA gave a more consistent response with maximum number of leaf responding (55%) at 0.087 mM and minimum (49%) at 1.312 mM. There was a sharp decrease in the frequency with 2,4-D. It was reported earlier that *P.* indica is very sensitive to 2,4-D *in vivo* condition (Motooka *et al.*, 2002) and this was expressed in the culture condition also. A general similarity was observed in the response pattern in the amount of callus induction at different concentrations of the hormone used. In all the three cases 5 mg/l was most effective and highest amount of callus (275 mg/l) was induced when IAA was supplemented in the medium. The size of the explant was directly proportional to the frequency of response, where immature young leaves failed to respond and turned brown presumably because of the juvenility factor, as these could not sustain the sterilization shock. When 1.13 μM NAA was supplemented to the medium, it was observed that soft white callus was induced at the cut edge of the explants and hard pinkish white and brown callus induced throughout the surface of the leaf explants. In all the three hormones, the amount of callus gradually reduced when the concentrations were increased above 5 mg/l.

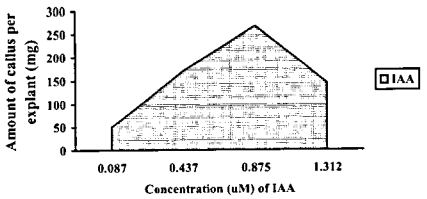
Variation among calli types was also observed when treated with the different hormones (Table 1 and Fig. 2). Soft creamish white calli was induced in MS supplemented medium whereas pinkish white hard granular calli were observed in IAA supplemented medium.

Shoot multiplication

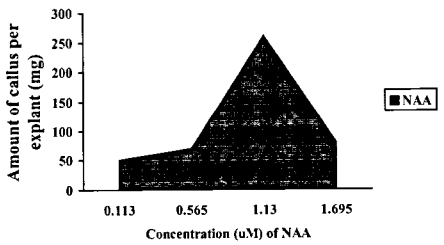
When stem pieces with 1 - 2 nodes were placed on the basal medium supplemented with BA and NAA, axillary bud multiplication giving rise to multiple shoots were achieved within 2 - 3 weeks. Shoot tips of regenerated shoots were continuously used to regenerate new shoots with periodic transfer to fresh medium resulting in a steady supply of healthy plants with increased production rate. At the later stage of the passage, normal roots were induced from the shoots, which appeared as clumps establishing exhaustion of BAP in this stage. It can also be presumed that the indigenous cytokinin level is high in *P. indica*, which resulted in the high rate of multiplication in a very low BAP supplemented medium. Of the various combination and concentrations of hormones used for shoot

Table 1. Effect of IAA, NAA, 2, 4-D, on callus induction from explants of *P. indica* (L.) Less., after 4 weeks of culture

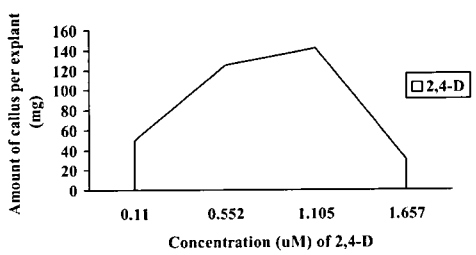
MS + Growth Regulators (μM)			Callus induction	
IAA	NAA	2,4-D	% of explants responding	Amount of callus (mg)
0	0	0	0	0
0.087	0	0	50	50
0.437	0	0	32.6	170
0.875	0	0	34	266.6
1.312	0	0	25	145
0	0.113	0	53	50
0	0.565	0	50	70
0	1.13	0	55	260
0	1.695	0	34	80
0	0	0.110	55	50
0	0	0.552	50	125
0	0	1.105	49	141.6
0	0	1.657	49	30



Effect of IAA on callus induction from *P. indica* (L.) Less leaf explants



Effect of NAA on callus induction from *P. indica* (L.) Less leaf explants



Effect of 2,4-D on callus induction from *P. indica* (L.) Less leaf explants

Fig. 2. Effect of IAA, NAA, 2, 4-D, on callus induction from explants of *P. indica* (L.) Less. after 4 weeks of culture.

multiplication, the following three combinations expressed satisfactory results:

Medium I - MS + 0.437 μM IAA
Medium II - MS + 0.437 μM IAA + 0.023 μM BAP
Medium III - MS + 0.437 μM IAA + 0.023 μM BAP + 1.73 μM GA_3.

The comparative analysis of the results obtained from the present study showed that though the maximum number of shoots were induced in Medium II, shoot length was greater in Medium III (Fig. 3, Fig. 1c). In Medium I, which is a cytokinin free medium, limited number of stunted shoots

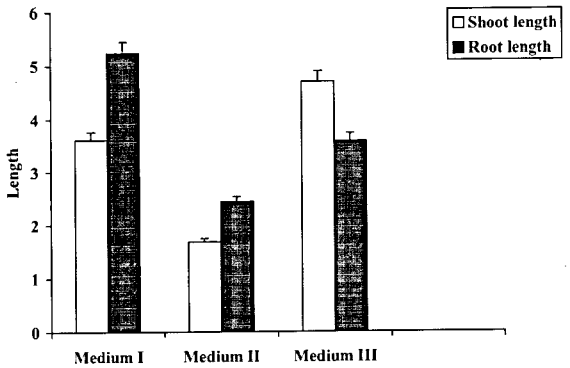


Fig. 3. Comparative analysis of the shoot length and root length of *Pluchea indica* (L.) Less. in three different concentrations and combinations of the growth regulators.

with thick broad leaves were induced, however, root proliferation was maximum compared to the other two media. A distinct difference in shoot length was observed in Medium III where internodes were longer and leaves smaller compared to those of Medium I and II. The result is in support with several other findings where effectiveness of combined application of cytokinin and auxin in shoot induction were demonstrated (Tideman and Hawker, 1982; Kukreja and Mathur, 1985; Mathur *et al.*, 1988; Luanratana, 1997).

It can therefore be concluded from the present observation that the selection of multiplication medium is to be done according to the requirement. For plant multiplication, with the aim to conserve the genotype, Medium II should be opted for. To obtain a greater root biomass for secondary metabolite extraction, Medium I is preferred. In the present protocol no special treatment was required for root induction in the *in vitro* grown shoots. Omission of a separate step for rooting shortened the total time schedule of micropropagation programme.

The significance of the proposed protocol is the selection of growth regulators in a particular ratio which stimulated direct multiplication of shoots, their growth, elongation and rooting. It was also noted that there was no decline in the rate of multiplication over a long period of nearly one

year. A very fast rate of multiplication of the plant was achieved here, as, from a single shoot section, thick clusters of multiple shoots were produced in each subculture. A similar type of growth pattern was also seen in *Phyllanthus amarus* (Bhattacharya *et al.*, 2003). Advantage of shoot tip/shoot section cultures has been realized earlier and successfully applied for rapid multiplication and long-term preservation of elite and desirable genotypes (Tanaka and Ikeda 1983; Sato *et al.*, 1987). Retention of regenerability and true-to-typeness in long term culture derived plants was suggested due to their genetic uniformity (Na and Kondo 1986), which is more assured by direct regeneration of plants than through intervening callus phase (Kukreja *et al.*, 1986).

Experiments are underway with *in vitro* regenerated roots to screen root clones with significant activity against inflammation, ulcer, diuretic, and diabetes in mice. This will further enhance the value of the present material for conducting therapeutic research with the plants.

Acute toxicity study

From the acute oral toxicity study carried out with Wistar albino rats, the LD_{50} value of MEPI leaves was found to be 2,825 mg/kg body weight.

Diuretic activity

Treatment with MEPI at doses of 100, 200 and 300 mg/kg, p.o. showed significant increase in urinary excretion of Na^+, K^+ and Cl^- in rats. The extracts also increased the volume of urine at all dose levels. The effect produced by MEPI at 300 mg/kg dose, was comparable to that of frusemide (20 mg/kg, p.o.). The results obtained have been shown in Table 2.

Evaluation of diuretic activity of MEPI suggested that it is an effective diuretic agent. Although MEPI caused significant diuresis in a dose dependent manner, the actual mode of action, which may be due to its effect either on loop permeability or reduction of antidiuretic hormone (ADH) secretion or inhibition of carbonic anhydrase enzyme, needs to be elucidated. Further investigation of the extract and screening of diuretic activity of phytoconstituents of the leaves of *P. indica* is necessary to reveal the exact mechanism of action.

This study addresses the need and possibility to advocate the conservation of biodiversity by means of plant tissue culture. Increased demand leads to higher pressure on the medicinal plant resource as it is chiefly sourced from the wild and common source and hence conservation of natural flora of the resource gains relevance. Issues like sustainable yield of medicinal plant leaves us with the option of *in vitro* regeneration as a mean to fall back on. There is also an urgent need today to concentrate on the research aspects for improvement of plants in terms of production of pharmacologically active secondary metabolites and plant tissue

Table 2. Effect of MEPI on diuretic activity in rats

Group	Treatment	Measured parameters			
		Total volume of urine ml/8 h	Total sodium (μmol/kg body wt./8 h)	Total potassium (μmol/kg body wt./8 h)	Total chloride (μmol/kg body wt./8 h)
I	2% v/v Tween 80 solution (10 ml/kg p.o.)	11.17 ± 0.19	319.83 ± 5.67	232.17 ± 7.83	1711.150 ± 8.02
II	Frusemide (20 mg/kg p.o.)	29.43 ± 0.29**	1955.50 ± 25.07**	877.83 ± 18.21**	6232.67 ± 9.42**
III	MEPI (100 mg/kg p.o.)	12.67 ± 0.21*	754.33 ± 7.78**	605.50 ± 7.18**	3530.00 ± 11.42**
IV	MEPI (200 mg/kg p.o.)	16.86 ± 0.31**	1029.00 ± 4.56**	751.00 ± 11.78**	5511.83 ± 27.95**
V	MEPI (300 mg/kg p.o.)	21.82 ± 0.29**	1681.67 ± 4.89**	1240.67 ± 7.17**	6246.17 ± 7.08**

Values are expressed as mean ± SEM (n = 6). *$P < 0.05$, **$P < 0.001$, compared to control (Dunnett's test).

culture offers major tools to achieve this objective. Again, pharmacology is a rapidly changing discipline of growing importance in today's health care system. Pharmacological studies hold promise for improving drug efficacy and lowering adverse effects in individual patients. Hence a coordinated multi-dimensional research aimed at correlating tissue culture studies to diuretic activities of the plant *P. indica* (L.) Less has been accomplished through this present communication. The present study provided scientific evidence in favor of the folklore use of the leaves of the plant *P. indica* as a diuretic agent. The plant merits further studies to elucidate the exact mechanism(s) of actions.

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