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RESEARCH ARTICLE

# In Vitro Regeneration and Bioactivity Evaluation of *Plumbago indica* for Enhanced Propagation and Plumbagin Production

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## Highlights

- Optimized *in vitro* regeneration enhances propagation efficiency of *P. indica*
  - Callus, shoot, and root development achieved under specific Plant Growth Regulator (PGR) combinations
  - Tissue-cultured plants show comparable antioxidant and toxicity activities
  - Environmental factors influence growth and plumbagin content in cultured plants
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# In Vitro Regeneration and Bioactivity Evaluation of *Plumbago indica* for Enhanced Propagation and Plumbagin Production

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## Abstract

*Plumbago indica*, a medicinal plant, is valued for its therapeutic potential due to secondary metabolites, particularly plumbagin. Conventional propagation methods are limited by slow growth and environmental constraints. This study aimed to optimize in vitro regeneration protocols for *P. indica* to enhance propagation efficiency, conservation, and secondary metabolite production. An indirect organogenesis protocol was developed, encompassing surface sterilization, callus induction, shoot and root development, acclimatization, and bioactivity assessment. The highest survival rate (83.34%) with minimal contamination was achieved using 0.3% Carbendazim® and 10% Clorox. Optimal callus induction occurred on Murashige and Skoog (MS) medium supplemented with 1.5 mg/L NAA and 2.5 mg/L BAP, yielding the highest callus weight ( $0.60 \pm 0.18$  g). Shoot induction was most effective with 4.0 mg/L BAP and 1.5 mg/L NAA, while the highest rooting was achieved in half-strength MS medium with 0.4 mg/L IBA, producing the longest roots ( $2.49 \pm 0.31$  cm) and the highest number ( $26.50 \pm 3.54$ ). Plants from Sabaragamuwa province exhibited the greatest height ( $104.60 \pm 8.35$  cm) and plumbagin content ( $42.90 \pm 3.25$  µg/mL), indicating environmental influence on growth and metabolite accumulation. Antioxidant activity (DPPH assay) showed IC<sub>50</sub> values of  $30.88 \pm 1.68$  µg/mL for tissue-cultured plants versus  $31.73 \pm 1.53$  µg/mL for conventionally propagated plants. Brine shrimp lethality assay indicated LC<sub>50</sub> values of  $37.46 \pm 2.90$  µg/mL (tissue-cultured) and  $44.53 \pm 0.69$  µg/mL (conventional). Anticancer activity (MTT assay) showed IC<sub>50</sub> values of  $117.18 \pm 2.93$  µg/mL (RD cells) and  $313.94 \pm 29.84$  µg/mL (Vero cells) for tissue-cultured plants, compared to  $103.07 \pm 3.35$  µg/mL (RD) and  $200.63 \pm 2.90$  µg/mL (Vero) for conventional plants. These findings highlight the potential of in vitro propagation for sustainable plant production and enhanced bioactive compound yield, supporting eco-friendly and economically beneficial strategies.

Key words: Anticancer Activity; Bioactivity Assay; In Vitro Indirect Organogenesis; Plumbagin; *Plumbago indica*

## INTRODUCTION

*Plumbago indica* L. (Plumbaginaceae) is a perennial herb or small shrub adapted to warm tropical climates. The species is believed to have originated in the Sikkim and Khasi hills of India and subsequently spread to neighbouring regions, including Sri Lanka. Owing to the high medicinal value of its roots, *P. indica* is now cultivated in many parts of the world (Jose et al., 2014). The plant is rich in diverse phytochemicals, including alkaloids,

flavonoids, saponins, glycosides, and tannins (Eldhose et al., 2013), which collectively contribute to a wide range of biological activities. Tannins are known for their wound-healing, antibacterial, anti-inflammatory, hypoglycemic, and antidiarrheal properties, while flavonoids function as potent free-radical scavengers and exhibit antidiarrheal, anti-inflammatory, hypoglycemic, anticancer, anti-allergic, and antimicrobial activities. Alkaloids, particularly heterocyclic indole derivatives, have been reported to possess anti-inflammatory, hypoglycemic, hypotensive,

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anticonvulsant, antiprotozoal, antimicrobial, and antimalarial effects (Tyagi & Agarwal, 2017).

The aerial parts of *P. indica* contain several bioactive compounds, including glycosides, amyrin, palmitic acid,  $\beta$ -sitosterol (Kumar et al., 2014), stigmasterol, campesterol, plumbagin, 6-hydroxyplumbagin, and plumbaginol. The red-colored inflorescences are rich in pigments such as pelargonidin, cyanidin, and delphinidin, which are widely distributed primary plant pigments, along with flavanols such as kaempferol. In addition, the flowers contain mono- and digalloylglucose derivatives. The roots are a major reservoir of quinones, including plumbagin, 6-hydroxyplumbagin, droserone, elliptinone, plumbagic acid, and lactones, as well as flavonoids such as flavonyl methyl ethers (ayanin, azaleatin), myricetin derivatives, ampelopsin tetramethyl ethers, carboxylic acids, alkaloids, aliphatic acids, and steroids such as  $\beta$ -sitosterol (Ariyanathan et al., 2010; Jose et al., 2014).

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is the principal bioactive compound isolated from the roots of *P. indica* (Gangopadhyay et al., 2010). It is a naturally occurring naphthoquinone of considerable commercial importance due to its broad spectrum of pharmacological activities (Silja et al., 2014). Given the extensive therapeutic potential associated with its phytochemical profile, *P. indica* has been traditionally used to treat a wide range of ailments. However, extensive harvesting has rendered the species increasingly rare in its natural habitats, highlighting the urgent need for alternative approaches to obtain valuable phytochemicals without further depleting natural plant populations.

Plumbagin is mainly exploited from the *P. indica* roots currently (Panichayupakaranant & Tewtrakul, 2002) which destroys the plant itself. Seed production is either low or non-existent, and the seeds produced are not viable. Therefore, shoot cuttings are used for the vegetative propagation of this plant (Yogananth & Basu, 2009). *P. indica* has slow growth and takes a considerably long period for roots to be ready for use in medicinal purposes (Panichayupakaranant & Tewtrakul, 2002). This plant is rapidly declining in its natural habitats in India due to overexploitation for commercial purposes (Gangopadhyay et al., 2010). Therefore, *in vitro* plant propagation methods are used as an alternate strategy for sustainable conservation and propagation of many medicinal plants, such as *P. indica* (Gangopadhyay et al., 2010).

## METHODOLOGY

*Plumbago indica* plants were obtained from an Ayurvedic herbal garden in Gampaha, Sri Lanka, and maintained in a shade house located at the Department of Botany, University of Sri Jayewardenepura (USJP). A potting mixture consisting of compost, sand, and topsoil in a 1:1:1 ratio was used, and the plants were watered regularly. These plants were maintained as the mother stock for obtaining explants for *in vitro* mass propagation and phytochemical

screening. The mother stock was treated with Albert solution (5.0 ml/L) twice a month and sprayed with a fungicide (Carbendazim 0.1%) fortnightly to protect against pests and pathogens, ensuring healthy and vigorously growing plants. A voucher specimen was prepared and authenticated by the National Herbarium at the Royal Botanic Gardens, Peradeniya, Sri Lanka.

## Culture media and growth room conditions

Murashige and Skoog (MS) (Murashige & Skoog, 1962) medium was used as the basal medium. The pH was adjusted to  $5.8 \pm 0.1$  before sterilization, and the medium was autoclaved at 121 °C and 15 psi for 20 minutes. The growth room temperature was maintained at  $25 \pm 1$  °C, and light was provided by cool white, fluorescent lamps, ensuring 3,000 lx of photosynthetically active radiation (PAR) under a 16-hour photoperiod. The humidity of the growth room was maintained at 50%.

## Statistical analysis

A Completely Randomized Design (CRD) was used for all experiments unless otherwise specified, and the cultures were randomized weekly. Statistical analysis was conducted using the statistical package “MINITAB” version 17. Analysis of Variance (ANOVA) was performed, and pairwise comparisons were made using Tukey’s test and the Least Significant Difference (LSD) method at a 5% confidence level. Values sharing the same letters were not significantly different. MS medium free of growth regulators was used as the control. Unless otherwise mentioned, there were at least fifteen replicates in all treatments.

## Optimization of surface sterilization

Initially, leaf explants were collected from the mother stock and washed with a few drops of liquid detergent, Teepol (0.01% w/v), for 5 minutes. The detergent was then completely removed by washing the explants vigorously under running tap water for 1 hour. Subsequently, the explants were transferred to a laminar airflow cabinet. Inside the cabinet, the explants were treated with 0.3% fungicide (Carbendazim) and 10% Clorox (domestic bleach containing NaOCl) mixed with two drops of TWEEN 20, for different exposure times of 5 minutes and 10 minutes. Each treatment step was followed by two successive washes with sterile distilled water. The explants were then treated with 70% ethanol for 30 seconds, followed by two additional washes with sterile distilled water. Finally, the surface-sterilized leaves were cut into approximately 1 cm<sup>2</sup> pieces and cultured on MS medium without growth regulators. At least twenty replicates were used for each treatment. Cultures were observed daily, and the percentage survival and contamination of explants were recorded after seven days of incubation. The best treatment was selected for further experiments.

### In vitro callus induction

Surface-sterilized immature *P. indica* internodes (1.0 cm length pieces) were placed on MS media supplemented with different combinations of Naphthalene acetic acid (NAA) (0.5 – 2.0 mg/L) and Benzyl aminopurine (BAP) (1.5 -3.0 mg/L). The swelling of the cut edges and color changes were observed daily over a period of eight weeks, and the mean fresh and dry weight of callus was measured after four weeks.

### Indirect shoot induction from calli

Three-week-old calli derived from leaf explants cultured on MS medium supplemented with 2.5 mg/L 2,4-D, 1.5 mg/L IBA, and 1.0 mg/L NAA were used for the experiment. Any trace of agar attached to the calli was carefully removed before transferring them to MS medium supplemented with varying concentrations of BAP (1.0–4.0 mg/L) and NAA (0.5–2.0 mg/L). Changes in callus color were observed over a period of eight weeks. A subset of six-week-old calli was sub-cultured into the same medium and incubated for an additional six weeks, while the remaining calli were maintained in the same medium without sub-culturing. Callus growth and shoot induction were observed after six weeks of incubation.

### Shoot elongation

Calli with induced buds from the previous experiment were transferred to MS medium supplemented with varying concentrations of Gibberellic Acid (GA<sub>3</sub>) (1.0–5.0 mg/L) or BAP (0.5–2.5 mg/L) separately for shoot elongation. Initial shoot heights were recorded before culturing on the shoot elongation media. The mean increment in shoot length was measured after eight weeks of incubation, based on the height of the tallest shoot bud in each shoot cluster.

### In vitro root induction

Well-grown *P. indica* multiple shoots (shoot height > 2.0 cm) obtained from the shoot elongation medium were carefully separated from the clusters after two months. The agar attached to the shoots was removed, and the separated shoots were transferred to half-strength MS medium supplemented with varying concentrations of Indole-3-butyric acid (IBA) (0.2–0.8 mg/L) for root induction. After eight weeks, the rooted plantlets were removed from the vessels, and the percentage of rooting response, the mean number of roots per shoot, and the mean root length (excluding roots shorter than 1.0 cm) were recorded.

### Acclimatization of in vitro propagated plantlets

Plantlets kept in the root induction medium for two months were carefully removed without damaging the roots. The roots were washed thoroughly with water to remove all traces of the culture media. They were then dipped in a fungicide solution (Captan 1.0 g/L) for five minutes and transferred to soaked coir pellets (Jiffy products, JIFFY

PRODUCTS S.L. (PRIVATE) LTD, Sri Lanka). The plantlets were covered with transparent polythene after watering. The covers were removed only for two to three hours daily. After two weeks, the covers were permanently removed when the plants stopped wilting during the exposure period (2-3 hours) to the outer environment. Survival percentage was observed over a period of four weeks. Healthy plants were then transferred into pots containing a soil mixture (topsoil: compost (1:1)) for further development, and survival percentage was monitored over a period of three months.

### Comparison of growth, survival percentage and Plumbagin content in roots of tissue-cultured *P. indica* plants grown across provinces of Sri Lanka

Two months old acclimatized tissue cultured plants were planted in four different provinces of Sri Lanka: Western (Gampaha- 7° 0' 25.59" N, 80° 3' 29.66" E), Sabaragamuwa (Rathnapura- 6° 42' 24.5" N, 80° 23' 10.4" E), North Central (Dibulagala- 7° 49' 29.4" N, 81° 05' 16.5" E) and North Western (Kurunagala- 7° 28' 51.2" N, 80° 24' 06.3" E). Plants were grown in natural soil with a one-foot distance between each plant. Mean plant height and mean number of branches were recorded at three-month intervals over a period of nine months. After nine months, plants were uprooted and dried under direct sunlight for two weeks until a constant weight was achieved. A 10.0 g sample of dried root powder was extracted with 100.0 mL of absolute ethanol using a Soxhlet apparatus for 4 hours at 50 °C. The excess solvent was evaporated using a rotary evaporator at 40 °C and the crude extract (3.0 mg) from each sample was dissolved in 3.0 mL of absolute ethanol before being subjected to HPLC analysis (Ultimate 3000, ThermoFisher Scientific, Germany). Three replicate extractions were performed for the roots obtained from each province.

An aliquot of 10.0 µL was injected into the High-Performance Liquid Chromatography (HPLC) machine. HPLC analysis was performed by using a C18 column (length 150.0 mm; inner diameter 4.6 mm; particle size 5.0 µm) (Ultimate 3000, ThermoFisher Scientific, Germany) with a diode-array detector set to a wavelength of 254 nm. Separation was carried out at 33 °C and the mobile phase consisted of 90: 10 mixtures of absolute methanol and deionized water at a constant flow rate of 1.25 mL/min. The resulting chromatograms were analyzed using the Plumbagin standard curve to calculate the Plumbagin content in plant roots grown in four different provinces.

### Comparison of bioactivities of tissue-cultured and conventionally propagated *P. indica* root extracts

#### Antioxidant activity

The free radical scavenging activity of crude extracts from tissue-cultured and conventionally propagated *P. indica* plant roots was investigated using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, as described by Fernando and Soysa (2014), with slight modifications to determine

the antioxidant activities. Different concentrations of crude extracts (1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60 µg/mL) were prepared in absolute ethanol. A volume of 200.0 µL of DPPH reagent (100.0 µM), prepared in absolute ethanol, was added to 100.0 µL of the test sample, and the mixture was kept in the dark for 30 minutes. DPPH (300.0 µL) alone was used as the positive control. Absorbance was measured at 517 nm using an ELISA plate reader (Multiskan Sky with Cuvette, ThermoFisher Scientific, USA). L-Ascorbic acid was used as the reference standard antioxidant. IC<sub>50</sub> values were determined by plotting a graph with percentage inhibition against concentration.

### Toxicity assay

The toxicity of tissue-cultured and conventionally propagated plant root extracts of *P. indica* was determined through the brine shrimp lethality assay. Artificial seawater was prepared by dissolving 27 g of sea salt in 1 L of distilled water (pH 7.9–8.1). Different concentrations (10.0–80.0 µg/mL) of crude root extracts were prepared in artificial seawater for the assay. Different concentrations (10.0 - 80.0 µg/mL) of crude extracts were prepared in artificial seawater. The extracts were initially dissolved in 99.9 % Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO 63178, USA), and the final concentration of DMSO in test samples was adjusted to 1% to prevent DMSO toxicity. Brine shrimp cysts (0.1 g) were added to a 500.0 mL conical flask, half-filled with artificial seawater (pH 7.9 - 8.1), and the flask was constantly aerated with an air pump while providing continuous light. Active nauplii were collected after 24 hrs.

$$\% \text{ Mortality} = \frac{(\text{No. of live nauplii in control} - \text{No. of live nauplii in treated})}{\text{Total number of nauplii in control}} \times 100$$

LC<sub>50</sub> values were determined by a graph plotting percentage mortality against the concentration of the plant crude extract.

### Anticancer activity by MTT assay

#### Cell culture

Cytotoxic studies were conducted using human Rhabdomyosarcoma (RD) cells, derived from the biopsy specimen of a 7-year-old female with pelvic Rhabdomyosarcoma previously treated with radiation and cyclophosphamide (Fernando et al., 2015), and African green monkey kidney (Vero) cells, derived from the kidney of an African green monkey (*Cercopithecus aethiops*) (Ammerman et al., 2008). The RD cells were preserved and maintained at the Department of Microbiology, Faculty of Medicine, University of Sri Jayewardenepura, while the Vero cells were preserved and maintained at the Department of Preclinical Sciences, Faculty of Medicine, General Sir John Kotelawala Defense University, Sri Lanka.

Preserved cells were added to a sterile Falcon tube containing

10.0 mL of cell culture medium. The culture medium was prepared by mixing 87% V/V Minimum Essential Medium (MEM), 10% V/V of heat-inactivated fetal bovine serum (FBS), 1% V/V of Penicillin-Streptomycin, and 1% V/V of L-glutamine. The pH of the medium was adjusted to 7.2–7.5 using 7.7 % sodium bicarbonate (1.0 -1.5 mL). The falcon tube was centrifuged at 1000 rpm for 10 min at 4 °C, and the supernatant was discarded. The cell pellet was resuspended in 1.0 mL of cell culture medium. Cells were then cultured in a flask containing 7.0 mL of culture medium and incubated at 37 °C in a 5% CO<sub>2</sub> incubator until a confluent monolayer was formed. Subculturing was performed, and exponentially growing cells were used for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

### Preparation of plant root extracts

The same crude extracts obtained from tissue-cultured and conventionally propagated plant roots, which were used in previous assays, were utilized to prepare the concentration series. For RD cells, a concentration series ranging from 20.0 to 120.0 µg/mL was prepared, while for Vero cells, the range was 20.0 to 640.0 µg/mL. The crude extracts were initially dissolved in DMSO to achieve a final concentration of 0.125% (v/v) and then further diluted in the culture medium to prepare the required concentrations.

### Cytotoxicity assay

Cell viability was determined based on the ability of viable cells to convert yellow-colored MTT into insoluble purple formazan crystals via mitochondrial dehydrogenase enzymes (Silva & Soysa, 2011). Confluent monolayers of RD cancer cells and Vero cells in T75 culture flasks were washed with 8.0 mL PBS to remove residual medium. After removing PBS, 6.0 mL of Trypsin-EDTA was added to the flask, followed by incubation at 37 °C for 1–2 minutes. Cells were observed under an inverted microscope, and gentle tapping was applied to detach the cells. Subsequently, 12.0 mL or more of culture medium was added to each flask to inhibit Trypsin activity. The cell suspensions were collected into 50.0 mL Falcon tubes and centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and 1.0–2.0 mL of complete culture medium was added to resuspend the cell pellets. The cell concentration was calculated, and a cell density of  $1 \times 10^6$  cells/mL was prepared from the stock cell suspensions of both RD and Vero cells.

For the assay, wells of a 96-well plate were filled with 100.0 µL of  $1 \times 10^6$  cells/mL cell suspension and 200.0 µL of culture medium. The plates were incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 24 h. The cells were then treated with varying concentrations of tissue-cultured and conventionally propagated plant root extracts (20 -120 µg/mL for RD cells, 20.0 – 640.0 µg/mL for Vero cells) in triplicate with a total volume of 300.0 µL per well. As controls, 0.125% DMSO was used as the negative control, and 0.083mg/mL cycloheximide (prepared by mixing 25.0 µL of 0.1% cycloheximide with 275.0 µL culture

medium) was used as the negative control. The plates were then incubated at 37 °C in a 5% CO<sub>2</sub> incubator for another 24 h. After incubation, the spent medium was removed, and the wells were washed twice with 300.0 µL of PBS. Subsequently, 100.0 µL of MTT solution (5.0 mg/mL in MEM) was added to each well. The plates were covered with Aluminum foil and incubated at 37 °C in a 5% CO<sub>2</sub> incubator for 3 h. Following the incubation period, the medium was carefully removed, and the Formazan crystals were dissolved using 100.0 µL of 99.9% DMSO. Absorbance was measured at 570 nm using the microplate reader. Cell viability was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

The 50% cytotoxic concentration (CC<sub>50</sub>) was defined as the concentration of the extract required to reduce cell viability by 50%.

The brine shrimp lethality assay was carried out according to the method described by Baravalia et al. (2012) with slight modifications. McCartney bottles were filled with 5.0 mL of different concentrations (10.0 – 80.0 µg/mL) of plant crude extracts (plant crude extraction, artificial seawater, and 1% DMSO) separately for tissue-cultured and conventional propagated plant root extracts. Potassium dichromate (P5271, Sigma-Aldrich, St Louis, MO 63178, USA) was used as the positive control, while artificial seawater with 1% DMSO alone was used as the negative control. Potassium dichromate (1.0 – 40.0 µg/mL) was prepared by dissolving in artificial seawater and 1% DMSO. Twenty active nauplii were placed in each McCartney bottle. Each test was carried out in triplicate. Test samples were kept 24 h under light, and survivors were counted using a stereomicroscope (MS1107, Micros, Austria). The percentage of mortality in each concentration was calculated using the equation below (Sahgal et al., 2010).

## RESULTS AND DISCUSSION

### Optimization of surface sterilization

Despite the use of various surface sterilants, both fungal and bacterial contaminations were observed throughout the experiments. The number of explants contaminated with fungi was higher than that of those contaminated with bacteria. After seven days of incubation, the treatment with 0.3% Carbendazim® for 10 minutes followed by 10% Clorox for 10 min (T4) resulted in the highest survival percentage (83.34%) and the lowest contamination percentage (16.66%) (Table 1).

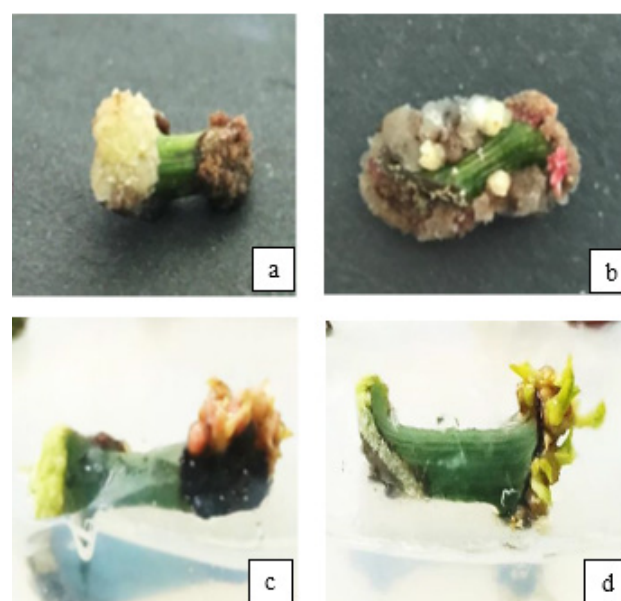
A study by Kumar et al. (2014) suggested that surface sterilization of *P. indica* nodal explants using running tap water for 5 min, followed by treatments with Bavastin (1%) and Tween 20 for 5-10 min and 0.1% (w/v) HgCl<sub>2</sub> for 3 min effectively controlled contamination. Similarly, (Bhadra et

al., 2009) reported successful surface sterilization of *P. indica* leaf and nodal segments using 0.2% (w/v) HgCl<sub>2</sub> for 10 min. HgCl<sub>2</sub> has also been widely used for surface sterilization in other studies (Bhattacharyya et al., 2015; Gopalakrishnan et al., 2009; Sharma & Agrawal, 2018; Sivanesan & Jeong, 2009). However, HgCl<sub>2</sub> was not used in the present study due to its high toxicity to humans and the environment, making it unsuitable for laboratory use.

### In vitro callus induction

Inter-nodal segments cultured on MS medium supplemented with different concentrations of NAA and BAP showed varying responses in callus production. Pinkish-white, friable callus induction was observed when NAA was supplemented at 1.0-2.0 mg/L in the presence of BAP at 2.5-3.0 mg/L concentrations (Figure 1). Lower concentrations of NAA and BAP did not induce any callus formation. Two weeks after culturing, the internodal segments began to enlarge, and callus induction occurred at the cut surfaces. The calli gradually grew to cover the entire surface of the explant within four to six weeks. Calli left in the same medium without subculturing for eight weeks turned from white to pale brown and eventually became fragile.

A significant difference in fresh and dry callus weights was observed among treatments with different PGR concentrations (Table 2). In this study, MS medium supplemented with 1.5 mg/L NAA and 2.5 mg/L BAP (T4) yielded the highest fresh callus weight (0.60±0.18 g) after four weeks of incubation. Meanwhile, MS medium supplemented with 1.0 mg/L NAA and 2.5 mg/L BAP (T3) produced the highest dry callus weight (0.10±0.02 g).



**Figure 1:** Callus and shoot induction from *Plumbago indica* internodal explants. Callus formation was induced on MS medium containing 1.5 mg/L NAA and 2.5 mg/L BAP (T4) at (a) four weeks and (b) six weeks. Shoot induction occurred on MS medium with 3.0 mg/L BAP at (c) four weeks and (d) eight weeks

**Table 1:** Effect of surface sterilization on contamination and survival of *Plumbago indica* explants

| Treatment Code | Exposure Time to 0.3% Carbendazim (min) | Exposure Time to 10% Clorox (min) | % Contamination | % Survival |
|----------------|-----------------------------------------|-----------------------------------|-----------------|------------|
| T1             | 5                                       | 5                                 | 58.33           | 41.67      |
| T2             | 5                                       | 10                                | 50.00           | 50.00      |
| T3             | 10                                      | 5                                 | 33.33           | 66.67      |
| T4             | 10                                      | 10                                | 16.66           | 83.34      |

**Table 2:** Callus induction and biomass (fresh and dry weight) of *Plumbago indica* internodal explants under different plant growth regulator (PGR) concentrations

| Treatment code | PGR Concentration (mg/L) |     | Callus induction    | Mean of fresh weight $\pm$ SD (g)             | Mean of dry weight $\pm$ SD (g)               |
|----------------|--------------------------|-----|---------------------|-----------------------------------------------|-----------------------------------------------|
|                | NAA                      | BAP |                     |                                               |                                               |
| T0             | 0.0                      | 0.0 | -                   | -                                             | -                                             |
| T1             | 0.0                      | 2.5 | -                   | -                                             | -                                             |
| T2             | 0.5                      | 2.5 | -                   | -                                             | -                                             |
| T3             | 1.0                      | 2.5 | White callus        | 0.58 $\pm$ 0.22 <sup>a</sup>                  | <b>0.10 <math>\pm</math> 0.02<sup>a</sup></b> |
| T4             | 1.5                      | 2.5 | White callus        | <b>0.60 <math>\pm</math> 0.18<sup>a</sup></b> | 0.09 $\pm$ 0.03 <sup>a</sup>                  |
| T5             | 2.0                      | 2.5 | White callus        | 0.57 $\pm$ 0.17 <sup>a</sup>                  | 0.08 $\pm$ 0.02 <sup>a,b</sup>                |
| T6             | 0.0                      | 3.0 | Multiple shoot buds | -                                             | -                                             |
| T7             | 0.5                      | 3.0 | Multiple shoot buds | -                                             | -                                             |
| T8             | 1.0                      | 3.0 | White callus        | 0.29 $\pm$ 0.05 <sup>b</sup>                  | 0.07 $\pm$ 0.02 <sup>b</sup>                  |
| T9             | 1.5                      | 3.0 | White callus        | 0.29 $\pm$ 0.10 <sup>b</sup>                  | 0.08 $\pm$ 0.02 <sup>a,b</sup>                |
| T10            | 2.0                      | 3.0 | White callus        | 0.45 $\pm$ 0.18 <sup>a,b</sup>                | 0.07 $\pm$ 0.03 <sup>a,b</sup>                |

Values marked with the different letters are significantly different with each other ( $P < 0.05$ )

Direct organogenesis from *P. indica* nodal segments has been reported on MS medium supplemented with 2.5 mg/L BAP and 1.0 mg/L NAA, 2.0 mg/L BAP and 2.0 mg/L NAA, 1.0 mg/L BAP and 0.1 mg/L NAA (Bhadra et al., 2009). In contrast, the present study observed direct shoot regeneration from internodal explants. Even though the production of multiple shoot buds from nodal segments is an abundant phenomenon in tissue culture, the induction of multiple shoots from internodal explants is rare. Internodal segments began to enlarge after two weeks, and pale pink protuberances were induced from the cut edges after four weeks, which gradually turned green within four to eight weeks. This type of multiple shoot bud induction from *P. indica* internodal explants has not been previously recorded in the literature.

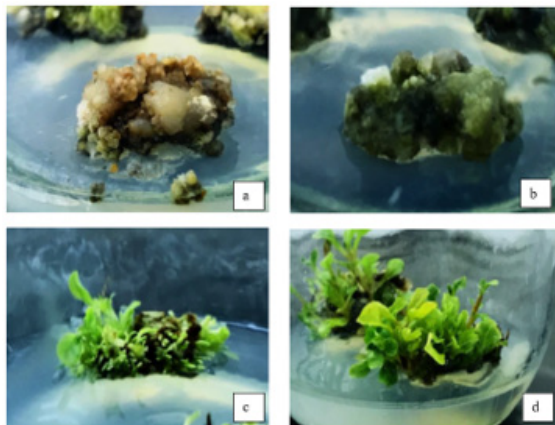
Direct shoot induction from internodal explants has been reported in other species. For example, *Mentha viridis* L. internodal explants produced a maximum of 36.7 $\pm$ 1.14 multiple shoots on MS medium supplemented with 3.5 mg/L BAP alone (Senthil & Kamraj, 2012). Similarly,

*Feronia limonia* L. internodal segments showed positive responses for direct shoot regeneration in the presence of BA at concentrations of 0.1-1.0 mg/L (Hiregoudar et al., 2005). These findings, along with the present study, suggest that BAP is the most effective PGR for direct organogenesis from internodal segments. Nitsch and Nitsch (1967) proposed that direct shoot production from internodal segments depends on both physical and chemical factors. In addition to the concentration of cytokinin, factors such as photoperiod length, concentrations of disaccharides, adenine, auxins, gibberellin, amino acids, amides, urea, and riboflavin also influence direct organogenesis from internodal explants.

#### Indirect shoot induction from calli

No shoot induction was observed in calli cultured on different BAP and NAA concentrations (25 combinations). Calli proliferated further and turned brown after eight weeks, except for those grown on MS medium supplemented with 4.0 mg/L BAP and 1.5 mg/L NAA. Calli grown on 4.0 mg/L

BAP and 1.5 mg/L NAA gradually became hard and pale green. When six weeks old, calli were sub-cultured again in the same medium for further proliferation. Pale brown calli were observed on all BAP and NAA concentrations except in MS medium supplemented with 4.0 mg/L BAP and 1.5 mg/L NAA. Calli in this medium produced green colored protuberances, six weeks after the first sub-culture. When the calli were kept in the same medium for twelve weeks, shoot elongation was observed (Figure 2).



**Figure 2:** Callus and shoot development in *Plumbago indica* internodal explants. (a) Browning of callus after eight weeks, (b) light green callus formation on MS medium with 4.0 mg/L BAP and 1.5 mg/L NAA after eight weeks, (c) shoot bud formation six weeks after subculturing, and (d) elongated shoots after twelve weeks

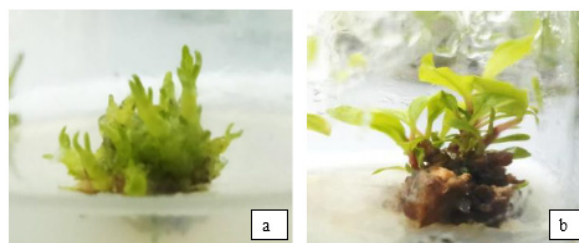
In literature, shoot induction from callus is also achieved by adding a high concentration of cytokinin with a low concentration of auxin. According to Kumar and Bhavanandan (1988) grey colored semi friable calli turned into hard, compact, and green calli within twenty days when cultured on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA. Those calli have produced shoot primordia when subsequently cultured on the same medium. The present study suggests a similar plant growth regulator combination, but at high concentrations for indirect shoot induction (4.0 mg/L BAP and 1.5 mg/L NAA).

Subsequent culturing on the same medium can induce shoots from calli. This phenomenon has been proven by previous studies (Kumar & Bhavanandan, 1988; Sharma & Agrawal, 2018), and the results of the current study. Sharma and Agrawal (2018) induced shoot buds from *P. zeylanica* callus by subculturing in MS media supplemented with different concentrations of thidiazuron (TDZ). In another study, Bhadra et al. (2009) kept Calli in the same medium for an extended period to undergo differentiation instead of sub-culturing in the same medium. Green nodular calli, produced in MS medium supplemented with 2.5 mg/L BAP and 2.0 mg/L NAA, were maintained in the same medium for 45 days to undergo differentiation. In the current study, the BAP concentration used for shoot induction from calli was higher when compared with the BAP concentrations of the above studies.

### Shoot elongation

Greenish shoot buds began to grow, and unopened leaves appeared after four weeks of incubation. Leaves gradually started to open within four to eight weeks, and the greenish color of the leaves intensified over time. Shoot height and the leaf surface size increased during an eight-to-twelve-week period (Figure 3). The highest mean shoot length increment ( $1.03 \pm 0.45$  cm) was observed in MS medium supplemented with 4.0 mg/L GA<sub>3</sub>, compared to other tested concentrations of GA<sub>3</sub> and BAP (Table 3). Among the tested BAP concentrations, the highest mean shoot length increment ( $0.79 \pm 0.38$  cm) was recorded in the presence of 0.5 mg/L BAP. Shoot elongation was also observed in the absence of plant growth regulator (control); however, the shoot length increment was significantly low.

The literature indicates that cytokines such as BA, in combination with GA<sub>3</sub>, can elongate *P. indica* shoot buds (Gopalakrishnan et al., 2009). The highest shoot length ( $2.0 \pm 0.0$  cm) was reported in MS medium supplemented with 0.12 mg/L BA and 0.5 mg/L GA<sub>3</sub>. The present study revealed that GA<sub>3</sub> alone can promote shoot bud elongation in vitro. However, the combined effect of GA<sub>3</sub> and BA was significantly greater. As observed in the current study, while growth regulator-free MS medium can elongate shoots, the mean shoot length increment was lower compared to the media containing PGR. Interestingly, in a study of Sharma and Agrawal (2018), *P. zeylanica* shoot bud elongation was successfully achieved using growth regulator-free MS medium.



**Figure 3:** Growth of *Plumbago indica* microshoots (MSB). (a) Immediately after culturing, and (b) after twelve weeks in MS medium supplemented with 4.0 mg/L GA<sub>3</sub>

### In vitro root induction

In vitro grown *P. indica* shoots (1.0-2.0 cm height) exhibited a 100% rooting response in all treatments, including the control. The highest mean number of roots ( $26.50 \pm 3.54$ ) and mean root length ( $2.49 \pm 0.31$  cm) were observed in half MS medium supplemented with 0.4 mg/L IBA after eight weeks (Table 4). Initially, the induced roots were friable and white, gradually becoming hard and brown over the eight weeks (Figure 4). Shoot height increased progressively along with root system development. Newly opened leaves broadened and turned dark green. It was observed that higher IBA concentrations (over 0.6 mg/L) inhibit root induction. A significant difference was noted in both the mean root length and the mean number of roots among the tested treatments.

**Table 3:** Shoot elongation of *Plumbago indica* on MS medium supplemented with varying concentrations of GA<sub>3</sub> or BAP

| GA3 Treatment |            |                                | BAP Treatment |            |                                |
|---------------|------------|--------------------------------|---------------|------------|--------------------------------|
| Treatment     | GA3 (mg/L) | Mean shoot length<br>± SD (cm) | Treatment     | BAP (mg/L) | Mean shoot length<br>± SD (cm) |
| T1            | 0.0        | 0.51 ± 0.21 <sup>b</sup>       | T1            | 0.0        | 0.35 ± 0.16 <sup>b</sup>       |
| T2            | 1.0        | 0.78 ± 0.43 <sup>ab</sup>      | <b>T2</b>     | <b>1.0</b> | <b>0.79 ± 0.38<sup>a</sup></b> |
| T3            | 2.0        | 0.71 ± 0.28 <sup>ab</sup>      | T3            | 2.0        | 0.57 ± 0.26 <sup>ab</sup>      |
| T4            | 3.0        | 0.87 ± 0.57 <sup>ab</sup>      | T4            | 3.0        | 0.56 ± 0.38 <sup>ab</sup>      |
| <b>T5</b>     | <b>4.0</b> | <b>1.03 ± 0.45<sup>a</sup></b> | T5            | 4.0        | 0.63 ± 0.30 <sup>ab</sup>      |
| T6            | 5.0        | 0.73 ± 0.18 <sup>ab</sup>      | T6            | 5.0        | 0.47 ± 0.18 <sup>b</sup>       |

Kumar et al. (2014) reported the best root induction (11.76±0.3) in half MS medium supplemented with 0.4 mg/L IBA, with a 90% rooting response. The present study confirms these findings, suggesting that half MS medium supplemented with 0.4 mg/L IBA is the most suitable growth regulator concentration for *in vitro* root induction of *P. indica*. However, in contrast to Kumar et al. (2014). Shoots grown in growth regulator-free half MS medium exhibited 40 % root induction; our study recorded a 100% rooting response in the growth regulator-free medium (control).

The optimal auxin concentration for root induction can vary between species, even among plants of the same genus. NAA (0.5 mg/L) was identified as the most effective plant growth regulator for *in vitro* root induction in *P. zeylanica* shoots compared to different concentrations of IAA and IBA (Sivanesan & Jeong, 2009). *In vitro* grown *P. indica* shoots exhibited an 80% rooting response, with a mean root number of 4.3±0.2 per shoot and a mean root length of 5.1±0.0 cm when cultured on half MS medium without any growth regulator (Gopalakrishnan et al., 2009). The available literature, along with findings from the current study, confirms that MS or half MS media without plant growth regulators can effectively induce root formation in *Plumbago* microshoots.

#### Acclimatization of *in vitro* propagated plantlets

A 100% survival rate was observed four weeks after planting tissue-cultured plantlets on coir pellets. However, the leaves of these plants withered easily due to poorly developed cuticles. When introduced into coir pellets, the plants produced small, fragile, and soft leaves. Covering the plants with polythene after planting helped reduce transpiration by increasing humidity around them and also provided protection from desiccation. Within two to four weeks, as the root system became well established in the coir pellets, the leaves began to expand. The green coloration of the leaves gradually increased, and plant height also improved within four to eight weeks. Acclimatized plants showed a 90% survival rate when transferred to pots containing a topsoil-to-compost (1:1) mixture. The surviving plants appeared healthy, and shoot

elongation was observed along with an increase in the number of leaves after one month (Figure 5).

Various acclimatization procedures have been used to introduce *in vitro* propagated *Plumbago* plantlets to the external environment. Rooted *P. zeylanica* plantlets were directly transferred to pots containing a mixture of soil, sand, and cow dung (1:1:1) immediately after removal from culture vessels. This method resulted in a 95% survival rate, with plantlets growing well in the greenhouse within 2–3 weeks (Rout et al., 1999). However, when *in vitro*-grown *P. indica* plantlets were introduced directly into a red soil, vermiculite, and farmyard manure (1:1:1) mixture, the survival rate dropped to 10% after three weeks of acclimatization (Gopalakrishnan et al., 2009). The present study revealed that using coir pellets before transferring plantlets to a soil-containing medium significantly improved the survival rate of *in vitro*-grown *P. indica* plantlets and can be suggested as a better acclimatization method.

Tissue-cultured plantlets typically have underdeveloped root systems that are less adapted to soil conditions and are susceptible to microbial infections and dehydration. Culturing plantlets on coir pellets before transferring them to soil allows the roots to adjust and develop further. Coir pellets have a high water-holding capacity, enabling immature roots to absorb water gradually while minimizing microbial damage to tender roots. As described by Lenora et al. (2012), tissue-cultured *P. indica* plantlets were introduced into polythene bags filled with a coir dust-to-sand (1:1) mixture before being planted in pots containing a soil, coir dust, and compost (1:1:1) mixture. However, the survival percentage of these acclimatized plantlets was not mentioned in the study. In another study, tissue-cultured *P. zeylanica* plantlets were first grown in culture bottles containing sterile soil, covered with a plastic cover, and kept in a culture room for two weeks before being transferred to pots containing a vermicompost-to-sand (50:50) mixture. These plants were maintained in a greenhouse before field transfer, resulting in a 90% survival rate (Ceasar et al., 2013).

**Table 4:** Root induction of *Plumbago indica* on half-strength MS medium supplemented with varying concentrations of IBA

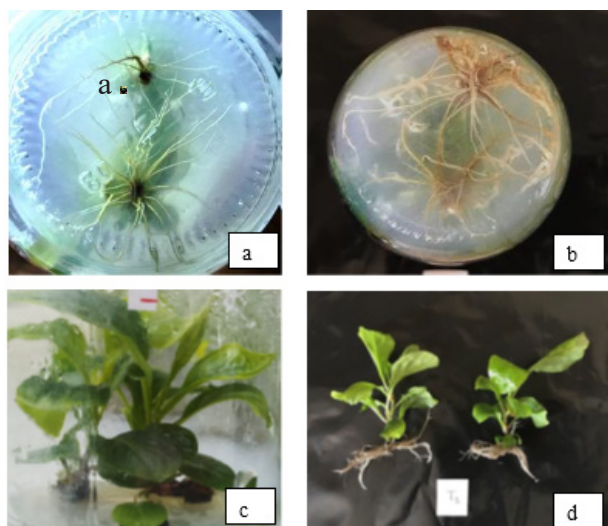
| Treatment | IBA (mg/L) | Mean number of roots $\pm$ SD | Mean shoot length $\pm$ SD (cm) |
|-----------|------------|-------------------------------|---------------------------------|
| T1        | 0.0        | 3.70 $\pm$ 1.64 <sup>c</sup>  | 1.48 $\pm$ 0.20 <sup>c</sup>    |
| T2        | 0.2        | 8.20 $\pm$ 1.87 <sup>b</sup>  | 1.85 $\pm$ 0.54 <sup>bc</sup>   |
| T3        | 0.4        | 26.50 $\pm$ 3.54 <sup>a</sup> | 2.49 $\pm$ 0.31 <sup>a</sup>    |
| T4        | 0.6        | 10.60 $\pm$ 2.72 <sup>b</sup> | 1.81 $\pm$ 0.51 <sup>bc</sup>   |
| T5        | 0.8        | 10.90 $\pm$ 2.89 <sup>b</sup> | 1.98 $\pm$ 0.58 <sup>b</sup>    |

#### Comparison of growth, survival percentage, and Plumbagin content in roots of tissue-cultured *P. indica* plants grown in different provinces of Sri Lanka

Tissue-cultured plants grown in the Western (Gampaha), Sabaragamuwa (Ratnapura), North Central (Dimbulagala), and North Western (Kurunegala) provinces showed 100% survival (Figure 6). Among these, plants grown in the Sabaragamuwa province exhibited the highest mean plant height (104.60 $\pm$ 8.35 cm), compared to those in the Western (94.40 $\pm$ 6.50 cm), North Central (34.40 $\pm$ 7.96 cm), and North Western (84.00 $\pm$ 6.32 cm) provinces. There was a significant difference in the mean plant height among plants grown in the four different provinces (Table 5). Tissue-cultured *P. indica* plants grown in the North Central province exhibited slow growth, stout stems, and small, thick leaves. Visual observations revealed that the width of the tuberous roots in these plants was considerably lower than that of those grown in the other provinces. The harsh environmental conditions of the North Central province, including high temperatures, low rainfall, dryness, and low soil nutrient content, were not favorable for *P. indica* growth. This species generally thrives in moist, well-

drained soils with temperatures ranging from 25 °C to 35 °C (Schmelzer & Gurib-Fakim, 2008). Plants grown in the North Central province had the highest mean number of branches (7.00 $\pm$ 2.00) compared to plants grown in other provinces. However, the mean number of branches across all four provinces did not show a significant difference.

HPLC results revealed that the tissue-cultured *P. indica* plant roots grown in the Sabaragamuwa province contained the highest plumbagin content (42.90 $\pm$ 3.25  $\mu$ g/mL), compared to those in the North Central (17.29 $\pm$ 5.23  $\mu$ g/mL), Western (35.81 $\pm$ 3.78  $\mu$ g/mL), and North Western (27.57 $\pm$ 4.00  $\mu$ g/mL) provinces. There was a significant difference in plumbagin content among roots grown in the four provinces. Plumbagin is one of the main secondary metabolites produced in *P. indica* roots. The production of secondary metabolites can vary depending on growth stage, age, flowering, locality, soil conditions, season,



**Figure 4:** Root induction of *Plumbago indica* on half-strength MS medium supplemented with 0.4 mg/L IBA. (a) Root formation after four weeks, (b) root development after eight weeks, (c) shoot growth in root induction medium after eight weeks, and (d) fully developed root system after eight weeks



**Figure 5:** Acclimatization and establishment of *Plumbago indica* plants. (a) After eight weeks on coir pellets, and (b) a potted plant in a topsoil: compost mixture (1:1)

and microbial interactions (Jose et al., 2014). No previous studies have reported variations in plumbagin production in *P. indica* roots under different environmental conditions. However, a previous study conducted in India on *P. zeylanica* found that plant roots grown in Mumbai and Damoh cities contained 0.30% and 0.37% plumbagin per 100 g of dry roots, respectively (Jain et al., 2014). This finding also supports the conclusion that plumbagin content can vary based on the locality of plant growth.

## Comparison of bioactivities of tissue-cultured and conventionally propagated *P. indica* root extracts

### Antioxidant activity

According to the results of the current study, the free radical scavenging activity of the extracts is concentration-dependent. The percentage inhibition of DPPH free radicals by tissue-cultured and conventionally propagated plant root crude extracts was plotted on the scatter chart (Figure 7). The tissue-cultured plant root extract had an  $IC_{50}$  value of  $30.88 \pm 1.68 \mu\text{g/mL}$ , while the conventionally propagated plant root extract had an  $IC_{50}$  value of  $31.73 \pm 1.53 \mu\text{g/mL}$ . There was no significant difference in free radical scavenging activity between conventionally propagated and tissue-cultured plant root extracts. The results indicate that the hydrogen-donating ability of L-Ascorbic acid (positive control) is greater when compared with the plant root extracts, with an  $IC_{50}$  value of  $3.04 \pm 0.14 \mu\text{g/mL}$ .

Ethanol extract of *P. indica* roots has higher free radical scavenging activity (82.34%) when compared with methanol (68.66%) and water extracts (63.36%) at a concentration of  $200.0 \mu\text{g/mL}$  (Nahak & Sahu, 2011). Therefore, ethanol is found to be a more effective solvent for extracting a higher content of phenolic compounds from *P. indica* roots. The  $IC_{50}$  value of the ethanolic extract of *P. indica* roots and ascorbic acid was recorded as  $18 \mu\text{g/mL}$  and  $8 \mu\text{g/mL}$ , respectively (Nahak & Sahu, 2011). In the current study, the *P. indica* root extract had an  $IC_{50}$  value of  $31.73 \pm 1.53 \mu\text{g/mL}$ . This difference might be due to the extraction procedure used in these two studies. The Soxhlet extraction method was used in both studies with different weights of dried root powder. Dried root powder (50.0 g) was used for extraction by Nahak and Sahu (2011), while 10.0 g was used in the current study. Therefore, the number and concentrations of phenolic compounds in the crude extract of Nahak and Sahu (2011) would be higher than those in the current study.

Ethanol is a better solvent for *P. indica* root extraction compared with hexane and chloroform. The number of phytochemical compounds dissolved in ethanol was higher, thus the ethanolic extract has higher free radical scavenging activity when compared with other solvents (Sujin et al., 2013; Ravali & Kumar, 2018). According to the observations of the above two studies, it is strongly suggested that ethanol is the best solvent for the extraction



**Figure 6:** Acclimatized 10-month-old tissue-cultured *Plumbago indica* plants. (a) Mature plants in Sabaragamuwa province, (b) roots in Sabaragamuwa province, (c) mature plants in Western province, and (d) roots in Western province

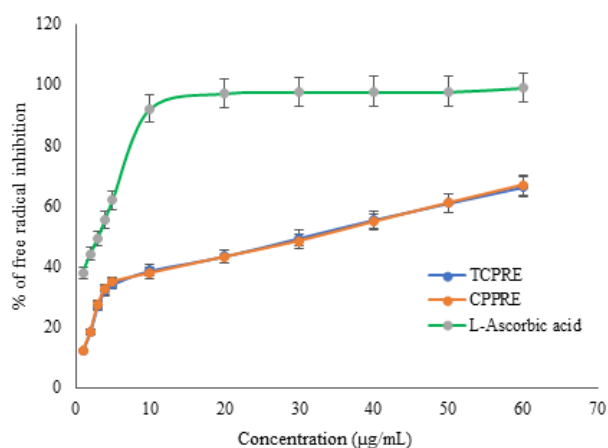
of *P. indica*. Methanolic extract of *P. indica* roots also contained phenolic and non-phenolic compounds that are mainly responsible for antioxidant activity. The  $IC_{50}$  value for the methanolic extract of *P. indica* roots was  $79.1 \mu\text{g/mL}$ , while ascorbic acid had a  $4.2 \mu\text{g/mL}$   $IC_{50}$  value (Eldhose et al., 2013). The amount of active phenolic compounds extracted into solvents can vary depending on the type of solvent used for extraction.

### Toxicity assay

The brine shrimp assay is a simple, rapid, and inexpensive preliminary toxicity assessment method used to detect the toxicity of plant extracts, heavy metals, pesticides, dental materials, and fungal toxins (Krishnaraju et al., 2005). DMSO was used to dissolve plant crude extracts in artificial seawater due to its partial solubility in water. The final percentage of DMSO in the assay volume was kept at 2% to avoid brine shrimp mortality due to DMSO toxicity (Baravalia et al., 2012). In the current study, the first trial was carried out with 2% DMSO in the assay volume.

**Table 5:** Growth and plumbagin content of tissue-cultured *Plumbago indica* plants grown in different provinces of Sri Lanka

| Provinces     | Mean height $\pm$ SD (cm)             | Mean number of branches $\pm$ SD    | Mean plumbagin content $\pm$ SD ( $\mu\text{g/mL}$ ) |
|---------------|---------------------------------------|-------------------------------------|------------------------------------------------------|
| Western       | $94.40 \pm 6.50^b$                    | $5.40 \pm 1.14^a$                   | $35.81 \pm 3.78^{ab}$                                |
| Sabaragamuwa  | <b><math>104.60 \pm 8.35^a</math></b> | $5.60 \pm 0.89^a$                   | <b><math>42.90 \pm 3.25^a</math></b>                 |
| North Western | $84.00 \pm 6.32^c$                    | $5.00 \pm 0.71^a$                   | $27.57 \pm 4.00^{bc}$                                |
| North Central | $34.40 \pm 7.96^d$                    | <b><math>7.00 \pm 2.00^a</math></b> | $17.29 \pm 5.23^c$                                   |



**Figure 7:** Dose–response curves showing DPPH free radical scavenging activity of *Plumbago indica* root extracts. Percentage inhibition was measured at different concentrations for L-ascorbic acid (standard), conventionally propagated plants, and tissue-cultured plants. (TCPRE: Tissue-cultured plant root extract, CPPRE = Conventionally propagated plant root extract)

However, the mortality percentage exceeded 30% even in the control. Therefore, the DMSO concentration was reduced to 1% to minimize its toxicity on brine shrimp.

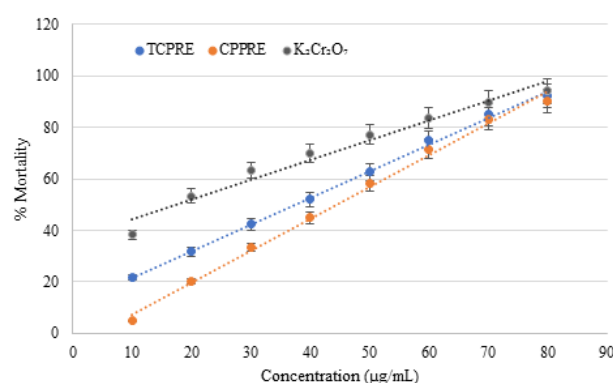
Brine shrimp were hatched in sterile artificial seawater prepared by dissolving 38 g/L sea salt (pH 8.5) under constant aeration for 48 hours (Krishnaraju et al., 2005). However, in the current study, brine shrimp eggs were hatched within 24 hours using artificial seawater prepared by dissolving 27 g/L sea salt (pH 7.9–8.1) under constant aeration for 24 hours. The percentage of brine shrimp mortality at different concentrations of conventionally propagated and tissue-cultured plant root crude extracts of *P. indica*, along with potassium dichromate, was plotted in the scatter chart (Figure 8). The  $LC_{50}$  value of the eight-month-old *P. indica* tissue-cultured plant root crude extract was  $37.46 \pm 2.90$  µg/mL, while the conventionally propagated plant root extract showed an  $LC_{50}$  value of  $44.53 \pm 0.69$  µg/mL. There was no significant difference between the  $LC_{50}$  values of tissue-cultured and conventionally propagated plant root extracts. Therefore, the toxicity of tissue-cultured and conventionally propagated plant roots can be considered equal. The  $LC_{50}$  value for the positive control (potassium dichromate) was  $17.21 \pm 3.54$  µg/mL.

Methanol extract of dried *P. indica* plant has shown a considerably low  $LC_{50}$  value (5.0 µg/mL) in the brine shrimp assay (Ibrahim et al., 2012). In the current study, the ethanolic extract of conventionally propagated *P. indica* roots showed an  $LC_{50}$  value of  $44.53 \pm 0.69$  µg/mL, which is significantly higher than the value reported by Ibrahim et al. (2012). Differences in extraction procedures, solvents used, plant age, and plant parts used for extraction may affect the phytochemical concentrations in the extracts. The Soxhlet extraction method was used in both studies. Ibrahim et al. (2012) used 97% methanol for extraction, while the current study used 99.9% ethanol. The extraction

time may also have influenced the  $LC_{50}$  values obtained; Ibrahim et al. (2012) extracted their samples for 18 hours, whereas the current study used a 4-hour extraction period. Furthermore, Ibrahim et al. (2012) used the whole plant for extraction without mentioning plant age, whereas the current study used eight-month-old roots.

Both the aerial parts and roots of *P. indica* contain a few common phytochemicals, such as plumbagin, 6-hydroxy plumbagin, and palmitic acid, which are primarily responsible for the plant's biological activities (Ariyanathan et al., 2010; Jose et al., 2014; Kaewbumrung & Panichayupakaranant, 2012, 2014; Kumar et al., 2014). Although both roots and shoots share the same genes responsible for phytochemical production, the activation of these genes in different plant parts may vary due to environmental and biological factors. The aerial parts of *P. indica* contain apigenin, cuteolin, 7-O-glycosides, amyirin, sitosterol, plumbaginol, stigmaterol, campesterol, pelargonidin, cyanidin, delphinidin, kaempferol, and mono- and digalloylglucose (Jose et al., 2014; Kumar et al., 2014). In contrast, the roots contain droserone, elliptinone, plumbagic acid lactone, flavonyl methyl ethers (ayanin, azaleatin), arachidyl alcohol, myricetin-3,3',5',7-tetramethyl ether, ampelopsin-3',4',5',7-tetramethyl ether, roseanoic acid,  $\alpha$ -naphthylamine, myricyl palmitate, and  $\beta$ -sitosterol (Ariyanathan et al., 2010; Jose et al., 2014; Kaewbumrung & Panichayupakaranant, 2012, 2014). Therefore, *P. indica* root and whole-plant extracts may contain different chemical compounds, contributing to their unique biological properties.

The dried powder of *P. indica* leaves and stems (185.0 g) was extracted with 1700.0 mL of methanol for ten days with intermittent shaking and stirring. The crude extract exhibited an  $LC_{50}$  value of 4.57 µg/mL in the brine shrimp lethality assay (Rafa et al., 2016). In the current study, 10.0 g of dried root powder was extracted with 100.0 mL



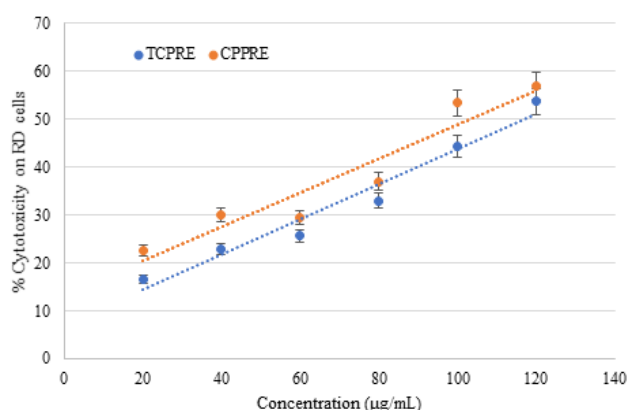
**Figure 8:** Dose–response curves showing brine shrimp lethality of *Plumbago indica* root extracts. Percentage mortality was measured at different concentrations for potassium dichromate (standard), conventionally propagated plant root extracts (CPPRE), and tissue-cultured plant root extracts (TCPRE)

of absolute ethanol. In the study by Rafa et al. (2016), a higher weight of dry plant material was used compared to the current study, and the extraction time was sixty times longer. Consequently, the content and concentration of phytochemicals in extracts obtained from these different extraction procedures may vary significantly.

### Anticancer activity by MTT assay

The anticancer activity of *P. indica* tissue-cultured and conventionally propagated plants was assessed using RD cells. The percentage of cytotoxicity exhibited by varying concentrations of the crude extracts was plotted in a scatter chart (Figure 9). The  $IC_{50}$  value for the tissue-cultured plant root extract (TCPRE) was  $117.18 \pm 2.93$   $\mu\text{g/mL}$ , whereas the conventionally propagated plant root extract (CPPRE) showed an  $IC_{50}$  of  $103.07 \pm 3.35$   $\mu\text{g/mL}$ . This indicates that CPPRE exhibited significantly higher anticancer activity on RD cells than TCPRE.

MTT assay is the most common method used to measure cell viability. Viable cells can convert yellow-coloured MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into purple-coloured, insoluble formazan crystals. Therefore, the number of live cells is proportional



**Figure 9:** Dose–response curve showing cytotoxicity of *Plumbago indica* root extracts on RD cells. Percentage cytotoxicity was measured at different concentrations for conventionally propagated plant root extracts (CPPRE) and tissue-cultured plant root extracts (TCPRE)

to the concentration of formazan (Soysa et al., 2018). RD cells are large, multinucleated, and spindle-shaped (Hinson et al., 2013). The Vero cell line is commonly used to assess the effects of different chemical compounds on mammalian cells at the molecular level (Ammerman et al., 2008). Vero cells are spindle-shaped under normal conditions but round off when exposed to toxic environments (Liao et al., 2009).

*P. indica* tissue-cultured and conventionally propagated plant root crude extracts were dissolved in DMSO due to partial water insolubility. DMSO is an organic, amphiphilic molecule widely used for drug delivery because of its strong interactions with phospholipids, which facilitate the efficient transport of drug molecules across the cell

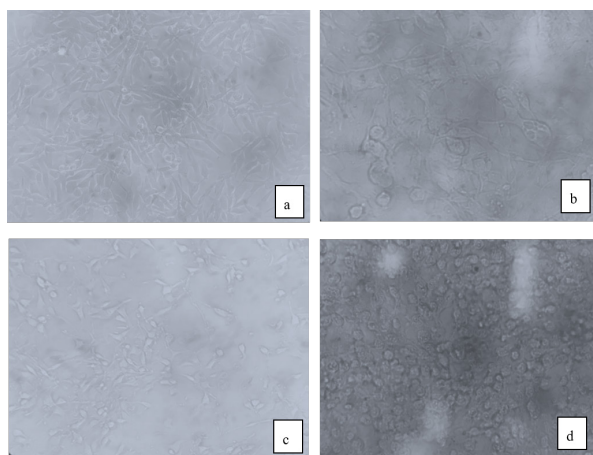
membrane (Nguyen et al., 2020). Ethanol and methanol are also commonly used to dissolve drugs or plant crude extracts that are partially soluble in water. However, ethanol can disrupt cellular physical activities by interfering with cholesterol structures in the cell membrane (Nguyen et al., 2020). Although these solvents exhibit toxicity in in vitro conditions, they are necessary for dissolving test compounds. Therefore, it is crucial to determine a safe solvent concentration before use. According to Nguyen et al. (2020), different cell types exhibit varying sensitivities to DMSO. Hence, a non-cytotoxic concentration should be identified for each cell line prior to conducting biological assays. DMSO concentrations ranging from 0.15% to 0.6% have been reported as non-toxic for HepG2, MDA-MB-231, MCF-7, and VNBRC1 cell lines.

No literature is available regarding the anticancer activity of *P. indica* plant extracts on RD cancer cells. However, studies have reported its effects on other cancer cell lines. The ethanolic extract of *P. indica* whole plant demonstrated anticancer activity against human cholangiocarcinoma (LC-6), human hepatocarcinoma (HepG2), and human laryngeal (Hep-2) cell lines, with  $IC_{50}$  values of  $77.79 \pm 14.31$   $\mu\text{g/mL}$ ,  $61.83 \pm 20.45$   $\mu\text{g/mL}$ , and  $40.50 \pm 13.52$   $\mu\text{g/mL}$ , respectively (Mahavorasirikul et al., 2010).

Plumbagin-5-O- $\alpha$ -L-rhamnopyranoside, a newly identified naphthoquinone glycoside from *P. indica*, exhibited inhibitory activity against HepG2 and MCF-7 cells, with  $IC_{50}$  values of  $7.93 \pm 0.15$   $\mu\text{M}$  and  $9.03 \pm 0.5$   $\mu\text{M}$ , respectively (Melk et al., 2021). Additionally, SK-MEL-28 cells (malignant melanoma) showed an  $IC_{50}$  of  $84.74$   $\mu\text{g/mL}$  when treated with the ethanolic extract of *P. indica* roots. Plumbagin, a major phytochemical in *P. indica*, exhibited an  $IC_{50}$  of  $30.48$   $\mu\text{g/mL}$  (Anuf et al., 2014), suggesting its significant role in the plant's anticancer activity. HPLC analysis from the current study revealed that the conventionally propagated plant root extract contained  $23.53 \pm 5.34$   $\mu\text{g/mL}$  of plumbagin, whereas the tissue-cultured plant root extract contained only  $12.90 \pm 2.42$   $\mu\text{g/mL}$ . The higher plumbagin content in CPPRE may explain its greater anticancer activity.

Morphological changes were observed in RD cancer cells treated with varying concentrations of the crude extracts and the positive control. Normally, RD cells exhibit large, multinucleated, spindle-shaped structures. Upon treatment with  $IC_{50}$  concentrations of the root extracts and cycloheximide, the cells became rounded (Figure 10).

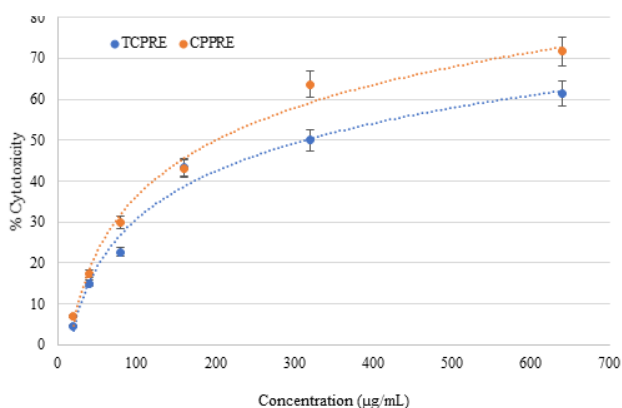
The percentage cytotoxicity exhibited at different concentrations of tissue-cultured and conventionally propagated plant root crude extracts on Vero cells was plotted in the scatter chart (Figure 11). The conventionally propagated plant crude extract showed an  $IC_{50}$  value of  $200.63 \pm 2.90$   $\mu\text{g/mL}$ , while the tissue-cultured plant root extract showed an  $IC_{50}$  value of  $313.94 \pm 29.84$   $\mu\text{g/mL}$ . These results indicate that the conventionally propagated plant root extract has higher cytotoxicity than the tissue-cultured plant root extract on Vero cells. There was a



**Figure 10:** Growth of RD cells after 24 h treatment with *Plumbago indica* root extracts (10× magnification). (a) Negative control, (b) 120.0 µg/mL tissue-cultured plant root extract (TCPRE), (c) 120.0 µg/mL conventionally propagated plant root extract (CPPRE), and (d) positive control

significant difference between the  $IC_{50}$  values of the tissue-cultured and conventionally propagated plant root extracts. The greater plumbagin content in the conventionally propagated *P. indica* plant root extract ( $23.53 \pm 5.34$  µg/mL) might explain its enhanced cytotoxic activity compared to the tissue-cultured plant root extract, which contained only  $12.90 \pm 2.42$  µg/mL plumbagin.

Quinones have been found to exhibit two key cytotoxic mechanisms in various cell systems. Under aerobic conditions, quinones undergo one-electron reduction by enzymes such as microsomal NADPH-cytochrome P-450 reductase or mitochondrial NADH-ubiquinone oxidoreductase, yielding semiquinone radicals. These radicals actively participate in redox cycling to generate superoxide anion and  $H_2O_2$ . Quinones are strong electrophiles that can react with thiol groups in proteins, as well as with glutathione (GSH). Menadione-induced cytotoxicity is triggered through GSH depletion.

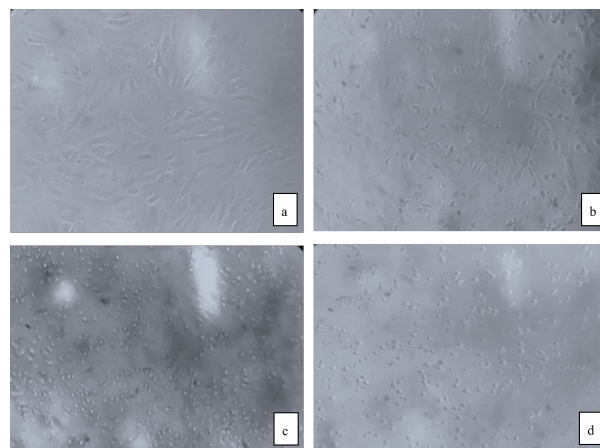


**Figure 11:** Dose–response curve showing cytotoxicity of *Plumbago indica* root extracts on Vero cells. Percentage cytotoxicity was measured at different concentrations for conventionally propagated plant root extracts (CPPRE) and tissue-cultured plant root extracts (TCPRE)

Plumbagin, a highly reactive quinone, mainly induces cytotoxicity through redox cycling,  $H_2O_2$  production, and GSH oxidation (Inbaraj & Chignell, 2004).

In Ayurvedic medicine, *P. indica* is used for medicinal purposes only after a purification process to reduce its toxicity. The roots are sliced into pieces and soaked in lime water ( $CaCO_3$ ) for 24 hours before being washed in hot water and dried. This purification significantly reduces the plumbagin content in *P. indica* roots (Akhilraj et al., 2019; Paul et al., 2013). However, there is no existing literature on the cytotoxicity of *P. indica* root extracts on Vero cells. Morphological changes in Vero cells were observed when treated with high concentrations of plant root extracts. Normally, Vero cells exhibit a triangular shape, but they round off when exposed to cycloheximide (0.083 mg/mL). In this study, similar morphological changes were observed when cells were treated with different concentrations of tissue-cultured and conventionally propagated root extracts (Figure 12).

The cytotoxicity of *P. indica* ethanolic root extract on the human gastric epithelial-17 (HGE-17) cell line was investigated by Paul et al. (2013). The purified *P. indica* root extract exhibited higher cytotoxicity ( $IC_{50} = 178.29$  µg/mL) compared to other species in the same genus, such as *P. zeylanica* ( $IC_{50} = 199.94$  µg/mL) and *P. auriculata*



**Figure 12:** Growth of Vero cells after 24 h treatment with *Plumbago indica* root extracts (10× magnification). (a) Negative control, (b) 320.0 µg/mL tissue-cultured plant root extract (TCPRE), (c) 320.0 µg/mL conventionally propagated plant root extract (CPPRE), and (d) positive control

( $IC_{50} = 278.59$  µg/mL). Additionally, the genotoxicity of the ethanolic extract of *P. indica* roots was evaluated using human lymphocytes. The extract showed significant genotoxicity at concentrations of 25–100 µg/mL, while a lower concentration (12.5 µg/mL) was neither genotoxic nor cytotoxic to human lymphocytes (Thitiorul et al., 2013). The present study demonstrated a concentration-dependent increase in both anticancer activity and cytotoxicity of *P. indica* tissue-cultured and conventionally propagated plant root extracts on RD and Vero cell lines.

*Plumbago indica* shows potential as an anticancer agent after reducing its cytotoxicity. The results revealed that the tissue-cultured plant root extract exhibited lower cytotoxicity ( $IC_{50} = 313.94 \pm 29.84 \mu\text{g/mL}$ ) on normal cells (Vero) while maintaining a considerable anticancer effect ( $IC_{50} = 120.74 \pm 2.93 \mu\text{g/mL}$ ) on RD cells. In contrast, the conventionally propagated plant root extract displayed both higher anticancer ( $IC_{50} = 103.48 \pm 3.35 \mu\text{g/mL}$ ) and cytotoxic ( $IC_{50} = 200.63 \pm 2.90 \mu\text{g/mL}$ ) activities.

Previous studies have already confirmed the anticancer and cytotoxic effects of plumbagin, the major phytochemical found in *P. indica* roots (Anuf et al., 2014; Powolny & Singh, 2008; Yan et al., 2013). The HPLC analysis from the current study revealed a significant difference in plumbagin content between the conventionally propagated and tissue-cultured plant root extracts ( $23.53 \pm 5.34 \mu\text{g/mL}$  and  $12.90 \pm 2.42 \mu\text{g/mL}$ , respectively). This variation in plumbagin content likely accounts for the differences in anticancer and cytotoxic activities observed between the two types of root extracts.

## CONCLUSION

This study successfully optimized in vitro regeneration protocols for *Plumbago indica*, enhancing propagation efficiency, sustainable conservation, and secondary metabolite production. The results demonstrate that tissue culture techniques can overcome the limitations of conventional propagation, with significant improvements in callus induction, shoot and root development, and acclimatization. Additionally, environmental factors were shown to influence both plant growth and plumbagin content. Bioactivity assays indicated that tissue-cultured plants exhibited antioxidant and toxicity profiles comparable to conventionally propagated plants, while showing slightly lower anticancer activity. These findings suggest that in vitro strategies can be effectively employed to enhance bioactive compound production. Overall, this work provides a foundation for the commercial production of *P. indica* via tissue culture, supporting sustainable, eco-friendly, and economically beneficial plant production strategies. Future research should focus on further optimizing growth conditions and increasing the yield of bioactive metabolites to meet commercial demands.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTION

Conceptualization: **H.A.S.A.P., W.T.P.S.K.**; Data collection, Statistical analysis: **H.A.S.A.P.**; Data interpretation: **H.A.S.A.P., M.D.K.M.G.**; Writing the first draft: **H.A.S.A.P., M.D.K.M.G., P.K.L.**; Manuscript editing and reviewing: **M.D.K.M.G., W.T.P.S.K.S., T.D.C.P.G.**; Funding acquisition: **W.T.P.S.K.S.**; Project administration: **W.T.P.S.K.S., T.D.C.P.G.**

## DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

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