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Development of an efficient in vitro system for micropropagation and regeneration of *Justicia adhatoda*, *Sida acuta*, *Pimenta dioica*, and *Premna integrifolia*

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Abstract

Medicinal plants have always been a non-exhaustible source of therapeutics throughout human history. Meanwhile, their in situ populations are under increasing anthropogenic pressure, specifically overharvesting and habitat destruction. Plant tissue culture is a powerful tool in biotechnological strategies of controlled selection, ex situ preservation and mass cloning of elite genotypes in medicine. The current study represents a study focusing on the development of an effective in vitro culture protocol of four important medicinal plant species: *Justicia adhatoda*, *Sida acuta*, *Pimenta dioica*, and *Premna integrifolia*. Induction of callus was achieved successfully by adding different concentrations of auxins and cytokinins to basal growth medium, especially 2,4-dichlorophenoxyacetic acid and Thidiazuron. Maximum callus formation was achieved in *J. adhatoda* (76.67%), *S. acuta* (88.33%), *P. dioica* (68.33%), and *P. integrifolia* (81.66%). Shoot regeneration was significantly enhanced by 6-benzylaminopurine, with responses of 62.22%, 73.33%, 65.55%, and 77.78% in the respective species. Root induction was effectively obtained with a combination of auxins, showing 72.22% in *P. integrifolia*, 79.99% in *S. acuta*, 84.44% in *J. adhatoda*, and 76.66% in *P. dioica*. The regenerated plantlets were acclimatized successfully under greenhouse conditions, confirming their survival and adaptability. The protocol showed very high efficiency in callus induction, shoot proliferation and root formation and therefore a valuable tool to be used in conservation, genetic improvement and sustained commercial exploitation of these medicinal species.

Keywords Plant tissue culture, Medicinal plants, Micropropagation, Plant growth regulators, Callus induction

1 Introduction

The use of medicinal plants for healing is as ancient as civilization itself. The search for natural pharmaceuticals has a long history, reflecting the deep-rooted interaction between humans and nature [1]. Almost every civilization has relied on medicinal plants



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as a source of medication. It is hard to trace the exact time when plants were first used for therapeutic purposes [2]. The Acanthaceae family member *Justicia adhatoda* L., commonly known as Vasaka or Malabar nut, is traditionally used to treat respiratory ailments, rheumatism, diabetes, and wound healing [3, 4]. *Sida acuta* Burm. f. (Malvaceae), with its long history in traditional medicine, is applied in the treatment of malaria, diarrhea, asthma, renal inflammation, and ulcers, and has shown anti-inflammatory, antimicrobial, and antioxidant activities [5, 6]. *Premna integrifolia* L. (Lamiaceae) is distributed across subtropical and tropical regions and is traditionally used for jaundice, menstrual disorders, liver ailments, and kidney diseases, while studies have confirmed its antioxidant, anticancer, hepatoprotective, and anti-hyperglycemic properties [7, 8]. *Pimenta dioica* L. (Myrtaceae), known as allspice, is cultivated in the Caribbean and Central America and exhibits antibacterial, anti-inflammatory, antifungal, and cytotoxic activities [9–11]. Growing worldwide demand for medicinal plants and their derivative products is now accompanied by unsustainable harvest levels and climate change impacts, which are causing sharp declines in biodiversity and loss of genetic diversity [12, 13]. This situation highlights the urgent need for sustainable propagation approaches.

Traditional methods of propagation, e.g., seed production and vegetative propagation, are often inadequate with respect to commercial requirements of genetic uniformity and quality of raw material [14]. In vitro micropropagation presents a sustainable and efficient solution for the mass propagation and conservation of these important plants. This method is extensively investigated for the micropropagation of several medicinal plants, as it enables rapid and extensive production of genetically identical plants within a constrained timescale and area [15]. Research on *Salvia officinalis* achieved 100% regeneration and developed stable callus cultures (95% viability) on Murashige and Skoog (MS) medium with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, yielding a 7.73-fold biomass increase [16]. In *Panax vietnamensis* immature embryo-derived somatic embryos had an efficiency of 44.33% on Schenk and Hildebrandt medium (7% sucrose), and the somatic callus culture was found to have saponins identical to those of wild plants [17]. The research on three Lamiaceae plants—*Thymus algeriensis*, *Rosmarinus officinalis*, and *Marrubium vulgare*—determined species-specific mixtures of indole-3-acetic acid and kinetin, enabling regeneration rates of 11–19% in these plants [18]. Additionally, *Ziziphora tenuior* tissue culture was demonstrated to increase antioxidant activity compared to wild plants [19]. Micropropagation of *Fritillaria cirrhosa* has been optimized with significant yield; achieving a 4-fold increase in the rate of shoot multiplication under the following conditions: basal medium with reduced naphthaleneacetic acid (NAA:0.2 mg/L) + 6-benzylaminopurine (BAP:1.0 mg/L) + Gibberellic acid (GA₃:1.0 mg/L) [20]. In parallel, robust *Stevia rebaudiana* genotype protocols demonstrate very high shoot multiplication rates (16.4–18.5 shoots/explant) that can be attributed to genotype-suitable hormone combinations [21]. Plant species that are threatened with extinction and are essential components of biodiversity like *Achillea fragrantissima* [22], *Clerodendrum indicum* [23] and *Maerua crassifolia* [24] have shown high success rates in experiments of tissue culture. However, the micropropagation of many medicinal species is still underdeveloped. While tissue culture techniques have been applied to some plants, efficient protocols are often missing or not fully optimized for species of interest. In vitro regeneration of Malabar nut is a poorly explored process despite being recognized as a rich source of alkaloid extracts [25]. Similarly, many medicinal plants do

not have a known reliable propagation system; *S. acuta* was one such plant until recently [26]. This is typical of most of the tropical medicinal plants whose mass multiplication and germplasm preservation require high-capacity systems that are currently insufficient compared with the demand. Despite its advantages, concerns have been raised regarding the genetic fidelity of micropropagated plantlets, emphasizing the need for genetic validation to confirm the stability of commercially propagated clones produced via in vitro methods [27]. Existing research shows a growing interest in addressing these obstacles through various channels. Significant advances include the addition of new media formulations and synergistic mixtures of growth regulators, the use of antioxidants to reduce browning during explant development, and advances in bioreactor design to achieve large-scale production [28]. Despite major technological breakthroughs, many medicinal species are still being propagated in nurseries without effective, reproducible in vitro propagation systems. Consequently, there is an urgent need to develop and refine in vitro regeneration techniques tailored to the biology of each species, to ensure sustainable production and conservation of valuable phytomedicines [29]. The current study aims to develop an effective micropropagation protocol of *J. adhatoda*, *S. acuta*, *P. dioica*, and *P. integrifolia*. This work aims to optimize conditions for rapid clonal multiplication and improve survival during acclimatization. The significance of this study is its potential to provide a sustainable platform for the large-scale production of these high-value plants, ensuring a consistent supply of disease-free material for the pharmaceutical industry and aiding in the conservation of their genetic resources.

2 Materials and methods

2.1 Plant materials

Healthy and viable seeds of *Justicia adhatoda* L. (1753) were collected from Sivapuri village, Tamil Nadu, India, and seeds of *Sida acuta* Burm.f. (1768) were collected from Annamalai Nagar, Tamil Nadu, India. Stem cuttings of *Pimenta dioica* (L.) Merr. (1947) were collected from Aanaikuppam, Cuddalore district, Tamil Nadu, India. Saplings of *Premna integrifolia* L. (1771) were obtained from Arya Vaidya Pharmacy Pvt. Ltd., Coimbatore, Tamil Nadu, India. All collected plant materials were cultivated under controlled greenhouse conditions at the Department of Molecular Biology and Biotechnology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, Chidambaram, Tamil Nadu, India. The plant materials were taxonomically identified and authenticated by Prof. L. Mullainathan, Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu, India. Voucher specimens were deposited at the Herbarium of the Department of Botany, Annamalai University, with the following accession numbers: *Sida acuta* (Acc. No. AU-978), *Justicia adhatoda* (Acc. No. AU-984), *Pimenta dioica* (Acc. No. AU-1127), and *Premna integrifolia* (Acc. No. AU-1128).

2.2 Explant preparation and culture condition

The explant preparation procedure was adopted from Kanmani Bharathi et al. [30], and the subsequent culture conditions were established and maintained with minor modifications. Surface sterilization was performed using 0.1% mercuric chloride for 2 min, followed by thorough rinsing with distilled water. Adjustments were made to the concentrations and combinations of plant growth regulators (PGRs), including 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), Indole-3-acetic acid (IAA),

1-naphthaleneacetic acid (NAA), and Indole-3-butyric acid (IBA). The medium pH was adjusted to 5.8. Cultures were maintained at a temperature of $25 \pm 2^\circ\text{C}$ and relative humidity of 65%. A 14/10-h photoperiod was maintained with the light intensity of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$. The sequential stages of in vitro regeneration from explant preparation to hardening are illustrated in Fig. 1.

2.3 Callus induction

For callus induction, 3 to 4 days old young pearl green leaves were surface sterilized and cut into 0.5 cm^2 explants. The explants were placed on MS medium with the abaxial side in contact with the medium, supplemented with varying levels of 2,4-D (0.5–2.5 mg/L), TDZ (0.5–1.0 mg/L), and IAA (0.5 mg/L) for initiation of callus and medium without PGR served as control. Cultures were subjected to incubation in the dark at $26 \pm 2^\circ\text{C}$ and subculturing was performed every 2 weeks for 4 weeks in the same medium. The callus initiation frequency for individual treatment was recorded after 8 weeks of culture. The nature of the callus development was categorized as “no callus initiation (N),” “white friable compact (WFC),” “translucent white friable compact (TWFC),” “milky white friable compact (MWFC),” “greenish white friable compact (GWFC),” and “white non-friable (WNF).”

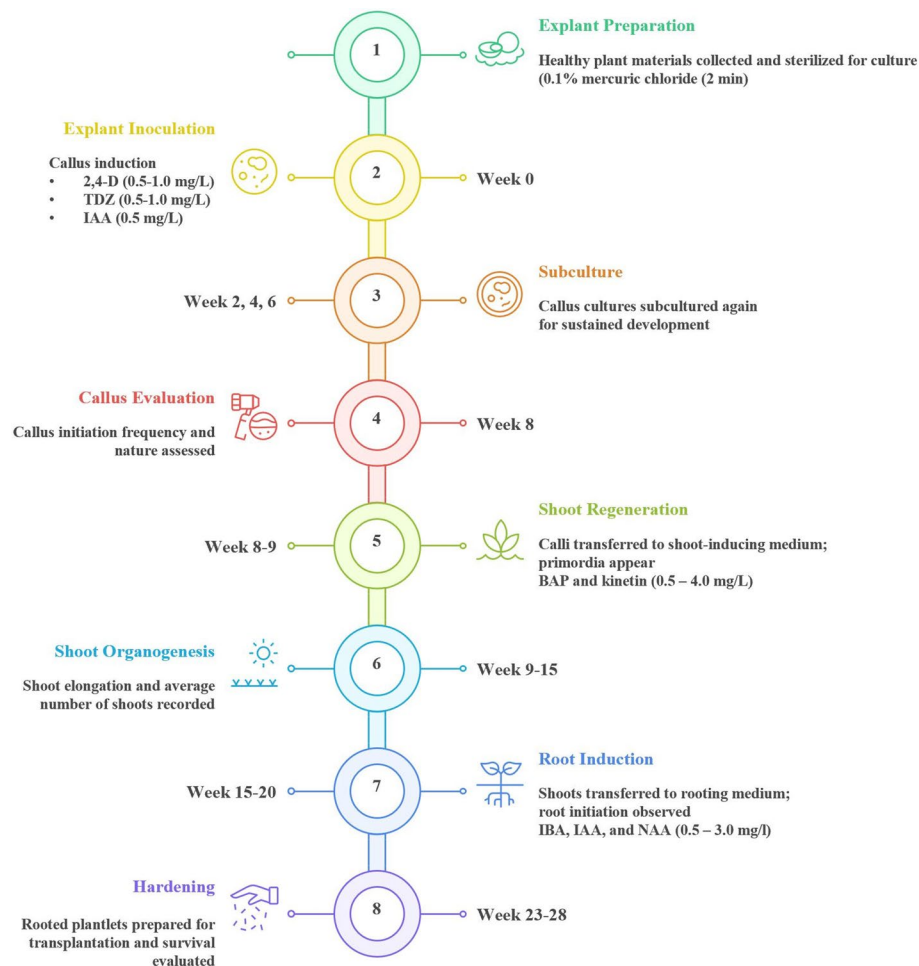


Fig. 1 Schematic representation of the in vitro regeneration protocol, showing sequential stages

$$\text{Callus formation (\%)} = \frac{\text{Number of explants showing callus development}}{\text{Total number of explants cultured}} \times 100$$

2.4 Shoot regeneration from callus

Well-established GWFC and TWFC calli were used for shoot regeneration. The calli were transferred to MS medium supplemented with either BAP (0.5–4.0 mg/L) or kinetin (0.5–4.0 mg/L). Cultures were subcultured biweekly for 5 weeks using the same medium to induce adventitious shoots. Green shoot primordia appeared within 3 weeks, followed by shoot elongation by week 5. Shoot organogenesis frequency and the average number of shoots per callus were recorded after 5 weeks of incubation. The nature of plantlets was summarized as “-: no shoots, +: stunted shoots, ++: normal shoots with leaves”.

$$\text{Shoot regeneration (\%)} = \frac{\text{Number of explants showing shoot organogenesis}}{\text{Total number of explants cultured}} \times 100$$

2.5 Root development

Regenerated shoots obtained from leaf-derived callus culture were placed on MS medium fortified with IBA, IAA, and NAA (0.5–3.0 mg/L) at different concentrations in various combinations for adventitious root initiation. Rooting responses in the cultures were observed after 3–5 weeks. At all stages, hormone-free MS medium was used as a control. Twenty treatments were evaluated in vitro for root development.

$$\text{Root regeneration (\%)} = \frac{\text{Number of explants showing root development}}{\text{Total number of explants cultured}} \times 100$$

2.6 Hardening

After five weeks, fully developed rooted shoots were removed from the medium and prepared for transplantation. The hardening procedure was adopted from Kanmani Bharathi et al. [30]. Plantlets were hardened in pots containing a soil mixture of sand: red soil: compost in a 2:1:1 ratio. Plantlet survival was evaluated over 30 days under both field and greenhouse conditions. Greenhouse hardening was carried out at 70–80% relative humidity (RH) and a temperature range of 25 ± 2 °C.

2.7 Statistical design and analysis

At each stage of micropropagation, 30 explants or regenerated plantlets were used per phytohormone concentration, and all experiments were conducted in triplicate for statistical analysis. All the tests were performed in a completely randomized design (CRD). Treatment averages were analyzed using SPSS version 25. Data were analyzed using one-way analysis of variance (ANOVA), and significant differences were determined by Duncan's Multiple Range Test (DMRT) at the 5% probability level [31]. The results were indicated as mean $\pm$ standard error (SE).

3 Result

3.1 Callus induction and development

Excised leaf explants were cultured on MS medium supplemented with different concentrations of 2,4-D (0–2.5 mg/L), TDZ (0–1.0 mg/L), and IAA (0.5 mg/L) for callus

induction. Callus formation was observed only after 4 weeks of culturing in *J. adhatoda*, *P. dioica*, and *P. integrifolia*, whereas in *S. acuta*, callus initiation occurred within 3 weeks. The effects of different concentrations of auxin (2,4-D), IAA, and cytokinin (TDZ) on callus induction from leaf explants are presented in Table 1. Among the tested treatments, the best callus induction response was recorded in *P. dioica* ($68.33 \pm 1.66\%$) and *S. acuta* ($88.33 \pm 1.67\%$) with T6 (Fig. 2c–g, o–s, and 3c–i, p, q). In contrast, maximum callus induction in *J. adhatoda* ($76.67 \pm 1.66\%$) and *P. integrifolia* ($81.66 \pm 1.67\%$) was observed in T4 and T8, respectively. The morphology of the induced calli varied among treatments and species. In *J. adhatoda* and *P. dioica*, calli obtained from T4, T6, and T8 were greenish-white, friable, and compact in appearance, respectively. In *P. integrifolia* and *S. acuta*, the calli were milky and translucent white. The other treatments produced only white, spongy calli with friability. No callus induction was observed in control medium. Importantly, none of the calli developed somatic embryos in the tested medicinal plants.

3.2 Adventitious shoot initiation and proliferation

The current study evaluated adventitious shoot initiation from leaf-derived callus cultured on MS medium with varying levels of BAP and kinetin. The maximum adventitious shoots from well-grown callus of *J. adhatoda* ($62.22 \pm 1.11\%$) and *S. acuta* ($73.33 \pm 1.92\%$) were recorded in T5 (3.0 mg/L BAP) (Fig. 2h, i, t–v), while higher shoot initiation responses were observed in *P. integrifolia* ($77.78 \pm 1.11\%$) and *P. dioica* ($65.55 \pm 1.11\%$) in T3 (1.0 mg/L BAP) (Fig. 3c–i, q). The highest regeneration response was obtained in the culture medium containing 1.0 and 3.0 mg/L BAP (Table 2). The maximum number of shoots per callus was recorded in *P. integrifolia* (3.87 ± 0.38) and *P. dioica* (3.62 ± 0.29) on T3. In contrast, T5 produced higher shoot numbers per callus in *J. adhatoda* (5.23 ± 0.43) and *S. acuta* (3.97 ± 0.19) (Fig. 4a). Shoot lengths were also influenced by BAP concentration: maximum shoot lengths of 5.12 ± 0.71 cm and 6.72 ± 0.53 cm were observed in *P. integrifolia* and *P. dioica* on T3, while *J. adhatoda* (3.20 ± 0.12 cm) and *S. acuta* (5.89 ± 0.37 cm) responded best on T5 (Fig. 4b). Other concentrations of BAP above 2.0 mg/L produced only a few shoots in *P. integrifolia* and *P. dioica*. Similarly, in *J. adhatoda* and *S. acuta*, shoot regeneration increased up to 3.0 mg/L BAP, but further increases significantly reduced regeneration efficiency. Both shoot number per callus and shoot length were negatively correlated with increasing BAP levels beyond the optimum treatments (T3 and T5). When kinetin was tested, comparatively lower shoot initiation was observed. The maximum shoot initiation response was recorded in *P. integrifolia* ($35.55 \pm 2.22\%$) and *P. dioica* ($37.78 \pm 2.94\%$) at 1.0 mg/L kinetin (T8). In *J. adhatoda* ($37.78 \pm 1.11\%$), maximum response occurred at 3.0 mg/L (T10), and in *S. acuta* ($42.22 \pm 2.22\%$) at 0.5 mg/L (T7). However, all kinetin-supplemented media generally produced stunted and abnormal shoots, except in *S. acuta*, where kinetin induced fewer shoots that appeared normal. In the control medium, no shoot proliferation was observed.

3.3 Rooting of adventitious shoots

The callus-derived shoots were transferred to root initiation medium containing half-strength MS nutrients with different concentrations of IAA, NAA, and IBA (0.5–3.0 mg/L) for root regeneration (Table 3). IBA and IAA combinations at concentrations

Table 1 Effect of auxin and cytokinins on callus development from leaf explants

Treatments	Phytohormones (mg/L)			<i>J. adathoda</i>		<i>P. integrifolia</i>		<i>P. dioica</i>		<i>S. acuta</i>	
	2, 4-D	TDZ	IAA	Percentage	Nature	Percentage	Nature	Percentage	Nature	Percentage	Nature
T1	–	–	–	0.00 ± 0.00 ^e	N	0.00 ± 0.00 ^h	N	0.00 ± 0.00 ^h	N	0.00 ± 0.00 ^g	N ¹
T2	0.5	0.5	0.5	38.33 ± 1.66 ^c	WFC	21.66 ± 1.67 ^f	WFC	30.00 ± 2.88 ^e	WNF	58.33 ± 1.66 ^d	WFC ²
T3	0.5	1.0	0.5	43.33 ± 1.67 ^c	GWFC ³	28.33 ± 3.33 ^{ef}	WFC	38.33 ± 1.67 ^{cd}	WNF	58.33 ± 1.66 ^d	WFC
T4	1.0	0.5	0.5	76.67 ± 1.66 ^a	GWFC	30.00 ± 2.88 ^e	WFC	33.33 ± 3.33 ^{de}	WFC	65.00 ± 2.88 ^{cd}	TWFC ⁴
T5	1.0	1.0	0.5	71.66 ± 1.67 ^a	GWFC	41.67 ± 1.66 ^d	WFC	38.33 ± 1.66 ^{cd}	WFC	68.33 ± 3.33 ^c	TWFC
T6	1.5	0.5	0.5	56.67 ± 3.33 ^b	WFC	53.33 ± 1.66 ^c	MWFC ⁵	68.33 ± 1.66 ^a	GWFC	88.33 ± 1.67 ^a	TWFC
T7	1.5	1.0	0.5	55.00 ± 2.88 ^b	WFC	55.00 ± 2.89 ^c	MWFC	60.00 ± 2.89 ^b	GWFC	78.33 ± 1.67 ^b	TWFC
T8	2.0	0.5	0.5	36.66 ± 1.67 ^c	WNF	81.66 ± 1.67 ^a	MWFC	43.33 ± 1.66 ^c	WFC	40.00 ± 2.88 ^e	WFC
T9	2.0	1.0	0.5	28.33 ± 1.66 ^d	WNF	70.00 ± 2.88 ^b	MWFC	35.00 ± 2.88 ^{de}	WFC	35.00 ± 2.88 ^e	WFC
T10	2.5	0.5	0.5	23.33 ± 3.33 ^d	WNF	13.33 ± 3.33 ^g	WNF	16.67 ± 3.33 ^f	WNF	16.67 ± 3.33 ^f	WNF ⁶
T11	2.5	1.0	0.5	23.33 ± 1.66 ^d	WNF	11.66 ± 1.66 ^g	WNF	8.33 ± 4.41 ^g	WNF	15.00 ± 2.89 ^f	WNF

Letter in superscripts denotes ranking by Duncan's multiple range test ($P < 0.05$). Each data indicates the mean ± SE of three independent tests

¹No callus
²White friable compact
³Greenish white friable compact
⁴Translucent white friable compact
⁵Milky white friable compact
⁶White non-friable

between 1.0 and 2.0 mg/L (T16 and T17) significantly increased rooting percentage from 60 to 70%, while concentrations above 2.0 mg/L resulted in a decreased response across the tested medicinal plants (*J. adhatoda*, *P. integrifolia*, *P. dioica*, and *S. acuta*). Treatments T16 and T17 both produced higher rooting responses but did not differ significantly from one other. When applied individually, IAA (T4) produced superior root initiation and development compared to IBA and NAA, achieving rooting percentages of $71.11 \pm 1.11\%$, $75.55 \pm 1.11\%$, and $61.11 \pm 1.11\%$ in *S. acuta*, *P. dioica*, and *J. adhatoda*, respectively (Figs. 2j, k and w, x and 3i–m and t–v). The combination of IBA (1.0–2.0 mg/L) with IAA (1.0–2.0 mg/L) significantly enhanced root regeneration compared to IAA alone, while also reducing the rooting period. Morphologically, thin, fragile roots were first observed at the cut ends of shoots within the first few days of inoculation, without callus formation. After 2 weeks, thickened primary roots with thin lateral branches were evident, and by 4 weeks, several long roots had developed. In the control medium, only thin, weak, hairy roots about 1 cm long were produced.

3.4 Hardening of regenerated plantlets

After successful regeneration, plantlets underwent hardening to optimize their survival rates. The regenerated plantlets obtained from indirect organogenesis were transplanted to pots containing a sand, soil, and compost mixture (2:1:1 ratio) for acclimatization. These pots were maintained under controlled culture conditions by covering them with polyethylene for 2 weeks. After this period, plantlets were gradually acclimatized to field conditions. The survival rates of plantlets under greenhouse and field conditions were analyzed. In greenhouse conditions, plantlets had a survival rate of $81 \pm 2.34\%$ (*J. adhatoda*), $78 \pm 1.97\%$ (*S. acuta*) (Fig. 2l, y), $86 \pm 4.56\%$ (*P. integrifolia*), and $93 \pm 4.97\%$ (*P. dioica*) (Fig. 3n, w). Under field conditions, survival rates were $70 \pm 2.43\%$, $67 \pm 2.11\%$, $71 \pm 2.96\%$, and $79 \pm 3.25\%$ for *J. adhatoda*, *S. acuta*, *P. integrifolia*, and *P. dioica*, respectively. No morphological irregularities were observed among the regenerated plants.

4 Discussion

This study establishes an efficient in vitro regeneration methodology, demonstrating that micropropagation is a crucial biotechnological method for large-scale propagation and conservation of valuable medicinal plant species. Conventional propagation techniques are frequently insufficient to meet pharmaceutical demand due to problems such as limited seed viability, slow growth rates, and genetic heterogeneity, while overharvesting and habitat loss create serious risks to many of these plants [32]. These results are consistent with recent literature observations, which commonly show that PTC has proven to be a stable and rapid alternative for producing genetically homogeneous, disease-free planting materials [33]. These methods offer a stable platform to perpetuate clonal propagation, making the supply of bioactive compounds more consistent, an issue of utmost importance to the phytopharmaceutical industry. Therefore, the development of this protocol not only provides a potential solution for sustainable production of the species in question but also contributes to the growing body of research aimed at protecting valuable phytopharmaceutical resources [34].

The present investigation demonstrates that 2,4-D at concentrations of 1.0–2.0 mg/L, in combination with 0.5–1.0 mg/L TDZ or IAA, was highly effective in promoting callus induction and development. These findings consistent with previous reports identifying

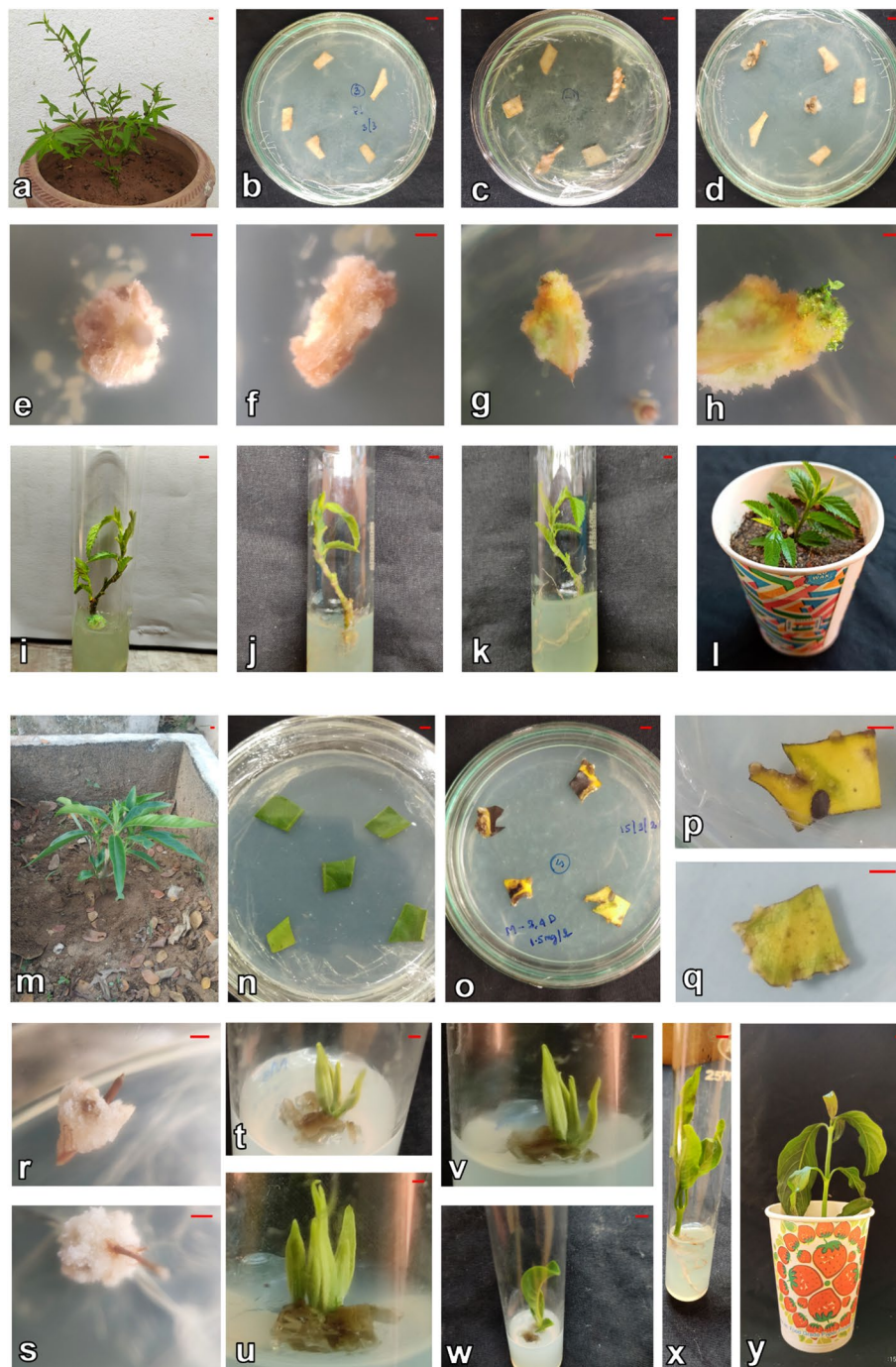


Fig. 2 **a–l** In vitro regeneration of *S. acuta*, **a** mother plants used for experiment, **b** leaf bits, **c**, **d** callus initiation after 2 weeks, **e**, **f** translucent callus mass, **g** greenish shoot buds developed from callus cells, **h** initiation of proliferated shoots, **i** elongated shoots, **j**, **k** root initiation and development, **l** rooted plants were acclimatized, **m–y** In vitro regeneration of *J. adhatoda*, **m** mother plants, **n** leaf bits, **o–q** callus initiation, **r–s** callus mass, **t–v** shoot development and multiplication, **w** shoots elongation, **x** rooting of callus derived shoots, **y** acclimatized plantlets. (Bar = 0.5 cm)

2,4-D as one of the most widely used auxins for callus formation [35] and further support its superiority over other auxins [36]. These results align with previous findings emphasizing the advantages of 2,4-D compared to other auxins like NAA or IAA in terms of efficiency and stability in tissue culture [37]. Specifically, 2,4-D has been shown



Fig. 3 a–n In vitro regeneration of *P. integrifolia*, a stock plants, b inoculated leaf explants, c–f callus development after 2 weeks c, 3 weeks d, e, after 4 weeks (f), g–i proliferated MWFC calli after 6 weeks, j–l shoot initiation and elongation, m root development, n acclimatized plantlets, o–w In vitro regeneration of *P. dioica*, o stock plants, p leaf bits, q callus development at cut ends of leaves, r shoot initiation from leaf derived callus, s, t shoot proliferation and elongation, u, v root induction from shoots, w regenerated plants acclimatized in pots. (Bar = 0.5 cm)

to accelerate callus production within 7–14 days, producing friable and embryogenic callus, whereas NAA typically prompts a more delayed response (14–21 days) and produces compact, slower-growing callus [38]. The superiority of 2,4-D for callus initiation is further supported by the fact that induction frequencies obtained with 2,4-D (70–100%) are higher than those obtained with IAA (50–83%) [39]. Furthermore, combinations of 2,4-D with cytokinins such as TDZ have been shown to significantly enhance induction efficiency. For instance, TDZ at 0.5 mg/L in combination with an equal concentration of 2,4-D produced 100% callus induction in strawberry cultures [40]. Similar

Table 2 Effect of various levels of cytokinins on shoot organogenesis from leaf-derived callus culture

Treatments	Phytohormones (mg/L)		<i>J. adathoda</i>		<i>P. integrifolia</i>		<i>P. dioica</i>		<i>S. acuta</i>	
			Percentage	Nature	Percentage	Nature	Percentage	Nature	Percentage	Nature
T1	–	–	0.00±0.0 ^h	–	0.00±0.0 ^h	–	0.00±0.0 ^g	–	0.00±0.0 ^h	– ⁷
T2	0.5	–	14.44±1.11 ^f	+	46.66±1.92 ^c	++	27.77±1.11 ^e	+	44.44±1.11 ^d	++ ⁸
T3	1.0	–	29.99±1.92 ^d	+	77.78±1.11 ^a	++	65.55±1.11 ^a	++	49.99±1.92 ^c	++
T4	2.0	–	48.89±2.22 ^b	++	57.77±1.11 ^b	++	57.78±1.11 ^b	++	67.77±1.11 ^b	++
T5	3.0	–	62.22±1.11 ^a	++	44.44±2.22 ^c	++	45.55±1.11 ^c	+	73.33±1.92 ^a	++
T6	4.0	–	41.11±1.11 ^c	++	38.89±1.11 ^d	+	34.44±2.22 ^d	+	45.55±1.11 ^{cd}	++
T7	–	0.5	9.99±1.92 ^g	+	26.67±1.92 ^f	+	24.44±1.11 ^e	+	42.22±2.22 ^d	++
T8	–	1.0	21.11±1.11 ^e	+	35.55±2.22 ^{de}	+	37.78±2.94 ^d	+	35.55±1.11 ^e	++
T9	–	2.0	32.22±1.11 ^d	+	32.22±1.11 ^e	+	26.66±2.92 ^e	+	34.44±1.11 ^e	+ ⁹
T10	–	3.0	37.78±1.11 ^c	+	22.22±1.11 ^f	+	15.33±1.11 ^f	+	20.00±1.92 ^f	+
T11	–	4.0	17.78±1.11 ^{ef}	+	13.33±1.92 ^g	+	11.11±1.11 ^f	+	9.99±1.92 ^g	+

Letter in superscripts denotes ranking by Duncan’s multiple range test ($P<0.05$). Each data indicates the mean ± SE of three independent tests

⁷No shoots
⁸Normal shoots with leaves
⁹Stunted shoots

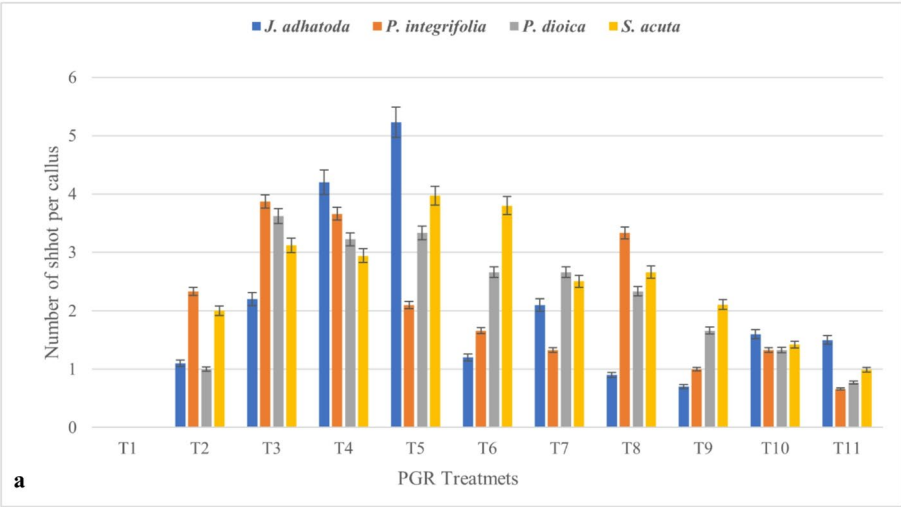


Fig. 4 a Number of shoots developed from callus from *J. adhatoda*, *S. acuta*, *P. integrifolia*, and *P. dioica*. **b** Mean length of proliferated shoots

trends were observed in this study, and comparable synergistic effects of 2,4-D and TDZ have been reported in *Melaleuca cajuputi* [41], *Cucurbita pepo* [42], *Lycium barbarum* [43], and *Paeonia lactiflora* [44]. The present observations showed that raising the concentration of 2,4-D from 0.5 to 1.0 mg/L produced a sharp increase in callus formation, whereas concentrations above 2.0 mg/L reduced induction efficiency. This dose-dependent response underscores the importance of maintaining optimal auxin–cytokinin ratios, as excessive auxin concentrations often inhibit morphogenesis. Although 2,4-D

Table 3 Effect of various combinations of auxins on root development in regenerated shoots

Treatments	Phytohormones (mg/L)			<i>J. adathoda</i>	<i>P. integrifolia</i>	<i>P. dioica</i>	<i>S. acuta</i>
	IAA	IBA	NAA				
T1	–	–	–	0.00 ± 0.0 ^l	0.00 ± 0.0 ^k	0.00 ± 0.0 ⁱ	0.00 ± 0.0 ^m
T2	0.5	–	–	25.55 ± 2.22 ^h	32.22 ± 2.22 ^g	27.78 ± 2.93 ^f	34.44 ± 1.11 ^h
T3	1.0	–	–	39.10 ± 1.92 ^f	66.66 ± 1.92 ^b	67.77 ± 1.11 ^b	64.44 ± 1.11 ^d
T4	2.0	–	–	61.11 ± 1.11 ^c	57.77 ± 1.11 ^c	75.55 ± 1.11 ^a	71.11 ± 1.11 ^c
T5	3.0	–	–	46.66 ± 1.92 ^e	41.11 ± 2.93 ^e	51.10 ± 2.22 ^c	54.44 ± 2.22 ^e
T6	–	0.5	–	19.10 ± 3.33 ^l	31.10 ± 2.94 ^g	41.11 ± 2.93 ^{de}	43.33 ± 1.92 ^f
T7	–	1.0	–	34.44 ± 1.11 ^g	38.88 ± 1.11 ^{ef}	38.88 ± 2.22 ^{de}	36.66 ± 1.92 ^{gh}
T7	–	2.0	–	26.66 ± 1.92 ^h	47.77 ± 1.11 ^d	34.44 ± 2.22 ^{ef}	31.22 ± 2.28 ^{hi}
T8	–	3.0	–	22.22 ± 1.11 ^{hi}	23.33 ± 1.92 ^h	29.99 ± 1.92 ^f	27.77 ± 1.11 ^j
T9	–	–	0.5	4.44 ± 2.22 ^{kl}	5.55 ± 1.11 ^{ij}	17.77 ± 2.93 ^g	17.77 ± 2.93 ^j
T10	–	–	1.0	13.33 ± 1.92 ^j	6.67 ± 3.33 ^{ij}	12.22 ± 2.93 ^{gh}	15.55 ± 1.11 ^{jk}
T11	–	–	2.0	7.77 ± 1.11 ^k	8.89 ± 1.11 ⁱ	9.99 ± 3.33 ^h	11.10 ± 2.93 ^{kl}
T12	–	–	3.0	5.55 ± 1.11 ^k	2.22 ± 1.11 ^{jk}	7.77 ± 2.93 ^h	6.55 ± 1.92 ^l
T13	0.5	1.0	–	39.99 ± 1.92 ^f	35.55 ± 1.11 ^{fg}	38.88 ± 2.22 ^{de}	42.22 ± 1.11 ^{fg}
T14	0.5	2.0	–	44.44 ± 1.11 ^{ef}	39.99 ± 1.92 ^{ef}	42.21 ± 2.93 ^d	45.55 ± 1.11 ^f
T15	1.0	1.0	–	53.33 ± 1.92 ^d	43.33 ± 1.92 ^{de}	56.66 ± 1.93 ^c	57.77 ± 2.94 ^e
T16	1.0	2.0	–	74.44 ± 1.11 ^b	75.55 ± 1.11 ^a	78.88 ± 2.94 ^a	87.77 ± 2.94 ^a
T17	2.0	1.0	–	84.44 ± 1.11 ^a	72.22 ± 2.22 ^a	76.66 ± 1.92 ^a	79.99 ± 1.92 ^b
T18	2.0	2.0	–	59.10 ± 1.92 ^c	47.77 ± 1.11 ^d	43.33 ± 1.92 ^d	36.66 ± 1.92 ^{gh}
T19	3.0	1.0	–	42.22 ± 1.11 ^{ef}	42.22 ± 2.22 ^e	34.44 ± 2.93 ^{ef}	31.11 ± 2.93 ^{hi}
T20	3.0	2.0	–	34.44 ± 1.06 ^g	24.44 ± 2.22 ^h	27.77 ± 2.93 ^f	18.33 ± 2.22 ^j

Letter in superscripts denotes ranking by Duncan's multiple range test ($P < 0.05$). Each data indicates the mean ± SE of three independent tests

with TDZ proved more effective than other combinations, the calli produced did not undergo somatic embryogenesis under the tested conditions, suggesting that additional factors may be required for embryogenic competence.

Somatic embryogenesis (SE) is a crucial micropropagation method in medicinal plants, enabling large-scale synthesis of genetically homogeneous propagules. High induction efficiencies have been reported in previous studies, including *Boswellia serrata* (89.6% response and 7–22 SEs per culture using TDZ + 2,4-D) [45], *Paramignya trimera* (100% callus induction and embryo formation confirmed by molecular markers) [46], and *Scaevola sericea* (7.5 SEs and 99% rooting) using TDZ (2.5 mg/L) [47]. These findings, which frequently achieve > 80% induction, demonstrate the dependability of optimized SE systems. However, the current investigation failed to produce somatic embryos in spite of successful callus development with 2,4-D and TDZ, demonstrating species-specific reactions and the crucial role that PGR balance plays in embryogenic competence. The tested therapeutic herbs may require additional optimization of culture conditions to fully realize their SE potential.

The present study further revealed that BAP was more effective than kinetin in inducing shoot regeneration from leaf-derived callus. This finding is in line with earlier studies emphasizing the dominance of BAP over kinetin in shoot development [48]. Specifically, optimal regeneration was obtained at 1.0 mg/L to 3.0 mg/L BAP, depending on species, which is consistent with Murali and Basavaraju's [49] reports, which showed effective shoot establishment at 1.0–1.5 mg/L BAP. Panigrahi et al. [35] also observed superior shoot initiation (98%) at 1.1 mg/L BAP, further validating these results. Comparable results have been reported in other medicinal plants, including *Aconitum ferox* [50], *Mondia whitei* [51], *Aegle marmelos* [52], *Labisia pumila* [53], *Sarcostemma acidum*

[54], and *Caralluma umbellata* [55]. Conversely, kinetin displayed limited efficacy in shoot regeneration. Except for *S. acuta*, which produced relatively better responses, most species developed stunted and abnormal shoots. These results strongly support the superiority of BAP in adventitious shoot induction.

IAA emerged as the most effective regulator for root induction, either alone or in combination with IBA, across all the studied medicinal plants. Comparable findings have been reported for *Lantana camara* [56], *Aconitum violaceum* [57], and *Lallementia royleana* [58], where IAA significantly enhanced rooting frequency and root number. For *Prunus africana*, Komakech et al. [59] also observed high rooting efficiency (75%) at 1.5 mg/L IAA. Although IBA was moderately effective, its performance was inferior to IAA, which consistently promoted higher rooting efficiency. These observations concur with Panigrahi and Patel [60], who recorded improved rooting with combined applications of IBA and NAA. Collectively, these results suggest that IAA plays a dominant role in root initiation compared to IBA under in vitro conditions. The successful acclimatization and hardening of regenerated plantlets are essential for their establishment under ex vitro conditions. In this study, high survival rates were achieved in both greenhouse and field environments, indicating the robustness of the acclimatization protocol employed. Similar high survival percentages following acclimatization have been reported for diverse medicinal plants [61, 62].

Genetic fidelity is essential in micropropagation, as clonal uniformity directly influences the reliability and commercial viability of therapeutic plants. While genetic fidelity analysis was not conducted in this study, prior research suggests that plants generated from tissue culture typically exhibit genetic stability; nonetheless, somaclonal variations may occur based on explant type, culture time, and hormonal conditions. Marker-based investigations, especially those employing Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSR), and Start Codon Targeted (SCoT) polymorphisms, demonstrate 95–100% fidelity in well-maintained systems, with direct regeneration processes generally displaying more stability than callus-mediated methods [63]. Several investigations indicate that ideal genetic homogeneity is achieved. In *Solanum khasianum*, nodal cultures on MS with 1.0 mg/L BAP + 0.5 mg/L NAA generated 12.4 shoots per explant, and fidelity analysis with 10 ISSR and 10 SCoT primers (90 amplicons, 300–3100 bp) indicated 100% monomorphic profiles [64]. Likewise, flower stalk explants in *Chlorophytum borivillianum* produced 15.27 ± 1.14 shoots on 2 mg/L BAP and 16.87 ± 1.53 roots on 0.5 mg/L NAA. Complete homogeneity among the 18 regenerants was confirmed using ISSR and SCoT analyses [65]. In *Ayapana triplinervis*, 2.0 mg/L BAP + 1.0 mg/L NAA generated 8.25 ± 0.75 shoots, and RAPD profiling (7 primers, 40 bands, 190–1600 bp) again demonstrated 100% fidelity [66]. However, minor genetic polymorphisms have been detected under certain conditions. In *Thunbergia coccinea*, direct organogenesis with 1.0 mg/L TDZ yielded 13.20 ± 0.37 shoots, although ISSR and RAPD studies (195 bands) demonstrated 99–100% similarity, with a 1% difference predominantly in callus-derived plants [67]. Additionally, ISSR profiling (10 primers, 39 bands, 200–3000 bp) showed 30 monomorphic and 9 polymorphic bands, with some primers displaying up to 75% polymorphism, which was primarily attributed to 2,4-D exposure. *Haplophyllum tuberculatum* showed more pronounced variability, with 0.5 mg/L BAP inducing 7.2 ± 0.4 shoots [68]. Overall, the findings suggest that tissue culture typically preserves genetic integrity (95–100% fidelity in most

cases), but callus-mediated regeneration and high auxin concentrations, particularly 2,4-D, can increase genetic variance. Recent findings have shown the dual effect of 2,4-D and TDZ in the induction of somaclonal variation during in vitro culture. Although these phytohormones are popular in producing somaclones or crop improvement, developmental abnormalities can also be induced when these phytohormones are used. As an example, 2,4-D and high subculture numbers have been linked to somatic embryogenesis defects [69] and TDZ and cytogenetic differences [70]. Molecular experiments show that long-term exposure to 2,4-D may cause changes in DNA methylation, causing genetic instability in species like *Cinchona officinalis* [71], and coffee [72]. These findings demonstrate the connection between phenotypic observations and the molecular and epigenetic mechanisms that cause phytohormone-triggered variation.

5 Conclusion

This study establishes, for the first time, standardized regeneration protocols for four medicinally important species: *J. adhatoda*, *P. dioica*, *P. integrifolia*, and *S. acuta*. Optimal callus induction was achieved with 2,4-D (1.0–2.0 mg/L) in combination with TDZ or IAA, with maximum responses of 88.33% in *S. acuta*, 81.66% in *P. integrifolia*, 76.67% in *J. adhatoda*, and 68.33% in *P. dioica*. Adventitious shoot proliferation was best supported by BAP, with *J. adhatoda* producing 5.23 shoots per callus at 3.0 mg/L, *S. acuta* 3.97 shoots at the same concentration, and *P. integrifolia* and *P. dioica* producing 3.87 and 3.62 shoots per callus, respectively, at 1.0 mg/L. Rooting was most effective with IAA (1.0–2.0 mg/L), yielding up to 75.55% in *P. dioica*, 71.11% in *S. acuta*, and 61.11% in *J. adhatoda*, while combinations with IBA further enhanced root quality. Acclimatization resulted in high survival rates, particularly 93% in *P. dioica* and 86% in *P. integrifolia* under greenhouse conditions. These procedures contain great potential for large-scale clonal development and conservation of high medicinal resources threatened by over-harvesting and habitat destruction. Moreover, they provide a dependable platform for the sustainable production of bioactive chemicals essential to the phytopharmaceutical operations. Future study should focus on increasing somatic embryogenesis, measuring genetic integrity through molecular markers, and scaling up regeneration via bioreactor systems to increase their economic utility. Collectively, this study adds to both conservation biology and applied plant biotechnology by proposing practical methods for the bulk propagation and sustainable exploitation of valuable medicinal plants.

Author contributions

M. Poornalakshmi, P. Gurumoorthy, M. Divya, and S. Sai Prathyusha Neelam performed the laboratory experiments. J. Kanmani Bharathi conducted the statistical analysis and initial manuscript preparation and editing. M. Prakash and R. Anandan contributed to the manuscript preparation and critical revision of the content.

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Data availability

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

The collection of plant material and the associated field survey were conducted in strict compliance with the provisions of the Biological Diversity Act, 2002, and all applicable local and national regulations of India. Prior formal permission for plant exploration, collection, and field investigations was obtained from the respective landowners and the competent botanical authorities, including the Department of Botany (Annamalai Nagar), the concerned Horticulture Department authorities of Aanaikuppam and Cuddalore District, and the authorized management of Arya Vaidya Pharmacy Pvt. Ltd. All research activities were carried out in accordance with ethical, legal, and biodiversity conservation standards.

governing plant-based research. This study did not involve human participants, and therefore consent to participate was not required.

Consent for publication

This study did not involve human participants, human data, or identifiable personal information; therefore, consent for publication was not required.

Competing interests

The authors declare no competing interests.

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