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IN VITRO CALLUS INDUCTION AND PRELIMINARY PHYTOCHEMICAL SCREENING OF *ARISTOLOCHIA INDICA* L.: A PROTOCOL DEVELOPMENT STUDY WITH SAFETY CONSIDERATIONS

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ABSTRACT
Aristolochia indica L. (Aristolochiaceae) is a medicinally significant yet threatened plant species widely used in traditional healthcare systems across South Asia. However, all Aristolochia species contain aristolochic acids (AAs), potent nephrotoxins and carcinogens that pose serious health risks. This study aimed to develop a reliable *in vitro* propagation protocol through callus induction and to characterize the major phytochemical constituents of leaf explants, with consideration of safety implications. Leaf explants (1.0 × 1.0 cm) were cultured on Murashige and Skoog (MS) medium supplemented with varying combinations of plant growth regulators, including 6-benzylaminopurine (BAP), naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D). Among all treatments tested, MS medium supplemented with 0.5 mg L^{-1} BAP and 0.4 mg L^{-1} NAA induced callogenesis in 78.3 ± 4.2% of explants with a mean fresh weight of 1.24 ± 0.18 g after 28 days of culture. Root organogenesis was subsequently achieved on MS medium containing 0.8 mg L^{-1} NAA, with a rooting efficiency of 65.7 ± 5.1%. Preliminary phytochemical screening of solvent extracts (acetone, methanol, petroleum ether, and distilled water) confirmed the presence of flavonoids, tannins, terpenoids, phenolic compounds, amino acids, alkaloids, and saponins. These findings demonstrate the feasibility of *in vitro* propagation of *A. indica* and underscore its considerable phytochemical diversity. However, the presence of aristolochic acids in this species necessitates careful risk assessment before any medicinal or conservation applications. Future work should focus on quantifying AA content in tissue-culture-derived material and on exploring the potential to develop AA-reduced or AA-free cultivars through biotechnological approaches.

Keywords: *Aristolochia indica, Callus induction, Micropropagation, Medicinal plant, Phytochemical screening, Organogenesis, Aristolochic acid, Plant tissue culture*

1. INTRODUCTION

1.1 Medicinal Plants and Conservation Biotechnology

Medicinal plants represent an indispensable reservoir of bioactive compounds and continue to play a pivotal role in both traditional and contemporary healthcare systems worldwide (Rao & Ravishankar, 2002). The World Health Organization estimates that approximately 80% of the global population relies on plant-based traditional medicine for primary healthcare needs (Farnsworth et al., 1985). However, the increasing demand for medicinal plants, coupled with habitat destruction, overexploitation, and climate change, has placed numerous species under severe threat of extinction (Chen et al., 2016). This conservation crisis has prompted the development of biotechnological approaches for the sustainable production and preservation of threatened medicinal plant species.

Plant tissue culture has emerged as one of the most effective biotechnological tools for the rapid multiplication, germplasm conservation, and secondary metabolite production of threatened medicinal taxa (Rout et al., 2000; Rao & Ravishankar, 2002). *In vitro* propagation offers several advantages over

conventional propagation methods, including year-round production independent of seasonal constraints, rapid multiplication rates, production of disease-free planting material, and the potential for genetic improvement through somaclonal variation or genetic transformation (Debergh & Read, 1991; George et al., 2008). The success of any *in vitro* propagation system is critically dependent on the type and concentration of plant growth regulators (PGRs) incorporated into the culture medium, as these compounds govern the balance between cell division, dedifferentiation, and organogenesis (Skoog & Miller, 1957; Murashige & Skoog, 1962).

1.2 The Genus *Aristolochia* and *A. indica*

The genus Aristolochia (Aristolochiaceae) comprises approximately 500 species distributed across tropical and temperate regions worldwide (González & Stevenson, 2000). *Aristolochia indica* L., commonly known as Indian birthwort or Ishwarmul, is a perennial herbaceous climber native to the Indian subcontinent, where it has been used for centuries in traditional Ayurvedic, Unani, and folk medicine systems (Chopra et al., 1956; Kirtikar & Basu, 1975). The plant has documented ethnobotanical applications in the treatment of snakebite, scorpion sting, inflammation, fever, gastrointestinal disorders, skin diseases, and as an abortifacient and emmenagogue (Nadkarni, 1976; Warrier et al., 1996).

Phytochemical investigations of *A. indica* have revealed a diverse array of bioactive compounds, including alkaloids, flavonoids, terpenoids, phenolic acids, and lignans (Sati et al., 2011; Desai et al., 2016). The species has demonstrated various pharmacological activities in preclinical studies, including anti-inflammatory, analgesic, antipyretic, antimicrobial, antioxidant, immunomodulatory, and wound-healing properties (Husain et al., 1992; Prabhu et al., 2009). However, sustained overexploitation for medicinal purposes, compounded by progressive habitat degradation and limited natural regeneration capacity, has placed natural populations of this species under serious threat, necessitating urgent conservation intervention (Pattar & Jayaraj, 2012).

1.3 Aristolochic Acid Toxicity: A Critical Safety Concern

Despite the traditional medicinal uses and documented pharmacological activities of Aristolochia species, a critical and non-negotiable safety concern must be addressed: all members of the genus Aristolochia contain aristolochic acids (AAs), a family of nitrophenanthrene carboxylic acids that are potent nephrotoxins, mutagens, and human carcinogens (Debelle et al., 2008; Grollman et al., 2007). Aristolochic acid I (AA-I) and aristolochic acid II (AA-II) are the predominant toxic compounds found in Aristolochia species (Michl et al., 2013).

The nephrotoxic and carcinogenic properties of aristolochic acids were first recognized in the early 1990s following an outbreak of rapidly progressive interstitial nephropathy in Belgium, subsequently termed “Chinese herbs nephropathy” or “Aristolochic Acid Nephropathy” (AAN) (Vanherweghem et al., 1993; Nortier et al., 2000). This syndrome is characterized by extensive interstitial fibrosis, tubular atrophy, and progressive renal failure, often necessitating dialysis or kidney transplantation (Debelle et al., 2008). Furthermore, patients with AAN exhibit an exceptionally high incidence of urothelial carcinoma, with up to 40-46% developing malignancies of the upper urinary tract (Nortier et al., 2000; Grollman et al., 2007).

The molecular mechanisms underlying AA toxicity have been extensively characterized. Aristolochic acids undergo metabolic activation by nitroreductases and cytochrome P450 enzymes to form reactive intermediates that covalently bind to DNA, forming characteristic aristolactam-DNA adducts (Arlt et al., 2002; Schmeiser et al., 2009). These DNA adducts induce A: T-to-T:A transversion mutations, particularly in the TP53 tumor suppressor gene, leading to carcinogenesis (Grollman et al., 2007; Poon et al., 2013). Additionally, aristolochic acids induce oxidative stress, mitochondrial dysfunction, and apoptosis in renal tubular cells (Balachandran et al., 2005; Shibutani et al., 2007).

Aristolochic acid nephropathy is not limited to the Belgian outbreak; it has been identified as the cause of Balkan Endemic Nephropathy (BEN), a chronic kidney disease affecting rural populations in southeastern Europe, where contamination of wheat flour with Aristolochia clematitis seeds has been documented (Grollman et al., 2007; Jelaković et al., 2012). Furthermore, epidemiological studies in Taiwan have revealed a strong association between the consumption of Aristolochia-containing herbal remedies and the

development of chronic kidney disease and urothelial carcinoma, with Taiwan exhibiting the highest incidence of urothelial carcinoma worldwide (Chen et al., 2012; Ng et al., 2017).

Specific to *A. indica*, phytochemical analyses have confirmed the presence of aristolochic acids in various plant parts. Michl et al. (2013) reported the detection of multiple aristolochic acid analogues in *A. indica* using high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS). Sudhakaran et al. (2014) quantified aristolochic acid I content in *A. indica* roots at 0.082% dry weight using high-performance thin-layer chromatography (HPTLC). These findings unequivocally demonstrate that *A. indica* contains toxic levels of aristolochic acids.

In response to the overwhelming evidence of toxicity, regulatory agencies worldwide have taken action to restrict or ban the use of Aristolochia-containing products. The United States Food and Drug Administration (FDA) issued warnings against the use of botanical products containing aristolochic acid in 2001 (FDA, 2001). The European Medicines Agency (EMA) prohibited the use of herbal medicinal products containing Aristolochia species in 2005 (EMA, 2005). Similar bans or restrictions have been implemented in numerous countries, including the United Kingdom, Germany, Australia, Canada, and Taiwan (Heinrich et al., 2009; Michl et al., 2013)

1.4 Tissue Culture of *Aristolochia* Species: Previous Work

Despite the recognized medicinal value and conservation needs of Aristolochia species, systematic studies on their *in vitro* propagation have been limited but growing. Several protocols have been developed for *A. indica* and related species over the past three decades.

Manjula et al. (1997) reported the first comprehensive *in vitro* regeneration protocol for *A. indica* using axillary shoot multiplication and organogenesis. Their study achieved high multiplication rates of 45-50 shoots per explant using nodal segments cultured on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Rooting was successfully induced on half-strength MS medium containing 1.0 mg L^{-1} IBA, and regenerated plantlets exhibited greater than 85% survival during acclimatization to ex vitro conditions. This pioneering work demonstrated the feasibility of micropropagation for conserving *A. indica*.

Soniya and Sujitha (2006) developed an efficient *in vitro* propagation protocol for *A. indica* using both direct and indirect regeneration pathways. Their study demonstrated that leaf explants cultured on MS medium supplemented with 0.8 mg L^{-1} BAP produced callus, which subsequently regenerated shoots on medium containing 0.8 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. Nodal explants showed 95% regeneration response, while leaf explants exhibited 85% response. This work highlighted the potential for using multiple explant sources for mass propagation.

More recently, Pattar and Jayaraj (2012) reported *in vitro* regeneration of plantlets from leaf and nodal explants of A. indica, emphasizing the species' threatened status and conservation needs. Their protocol achieved callus induction with 0.8 mg L^{-1} BAP, shoot induction with 0.8 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, and root induction with 0.8 mg L^{-1} NAA, demonstrating reproducible regeneration from both explant types.

Shah et al. (2013) investigated micropropagation of *A. indica* using various explant types and PGR combinations, contributing to the growing body of knowledge on optimal culture conditions for this species.

Nehra et al. (2017) compared the responses of different culture media to *in vitro* plant regeneration using different explants in A. indica, providing insights into medium optimization.

Importantly, Dey et al. (2021) published an advanced protocol that incorporated genetic fidelity assessment using molecular markers (RAPD and ISSR) to confirm clonal stability of micropropagated plants, addressing a critical concern in conservation biotechnology. Their study also included quantification of aristolochic acid content in tissue culture-derived material, representing a significant advancement in addressing safety concerns associated with Aristolochia propagation.

Related Aristolochia species have also been successfully micropropagated. Remya et al. (2013) developed protocols for Aristolochia tagala, including the production of artificial seeds for germplasm conservation. Saidi et al. (2010) reported indirect organogenesis in Aristolochia longa. These studies collectively demonstrate that Aristolochia species are amenable to *in vitro* propagation, though optimal protocols are species-specific and require careful optimization of PGR combinations.

1.5 Rationale and Objectives of the Present Study

While previous studies have established the feasibility of *in vitro* propagation of A. indica, several knowledge gaps remain. First, there is limited information on the comparative effectiveness of different auxin-cytokinin combinations for callus induction from leaf explants, particularly regarding the use of 2,4-D versus NAA as auxin sources. Second, the phytochemical composition of in vitro-derived callus tissue has not been comprehensively characterized, and it remains unclear whether tissue culture conditions influence secondary metabolite profiles. Third, most previous studies have focused on direct shoot regeneration from nodal explants, with less emphasis on indirect organogenesis via callus culture, which may offer advantages for large-scale propagation and somaclonal variation studies.

The present investigation was therefore undertaken with the following specific objectives:

1. To establish a preliminary callus induction protocol from leaf explants of *A. indica* using various combinations of plant growth regulators (BAP, NAA, IBA, and 2,4-D).
2. To evaluate the effectiveness of different PGR combinations for subsequent root organogenesis from callus tissue.
3. To conduct preliminary phytochemical screening of in vitro-derived leaf tissue to characterize the major secondary metabolite classes present.
4. To discuss the implications of aristolochic acid content for the safe application of this protocol in conservation and medicinal plant production.

This study represents a preliminary investigation into callus-based propagation of *A. indica*, with the ultimate goal of contributing to the conservation of this threatened medicinal species while acknowledging and addressing the critical safety concerns associated with aristolochic acid content.

2. MATERIALS AND METHODS

2.1 Plant Material and Explant Source

Healthy, disease-free specimens of *Aristolochia indica L.* were collected from the campus of Union Christian College, Aluva, Kerala, India (9.9312° N, 76.3503° E) during the monsoon season (June-July 2020). The plant material was authenticated by comparison with herbarium specimens at the Department of Botany, and a voucher specimen (UCC/BOT/AI/2018/01) was deposited in the departmental herbarium for future reference. Young, fully expanded leaves from actively growing vines were selected as explant sources. Leaves were harvested in the early morning hours (6:00-8:00 AM) to minimize physiological stress and maximize explant viability.

2.2 Surface Sterilization Protocol

Leaf explants were processed under aseptic conditions in a laminar airflow cabinet (Labtech, India). The surface sterilization protocol was optimized through preliminary trials and consisted of the following sequential steps:

1. Leaves were first washed thoroughly under running tap water for 15 minutes to remove surface debris, dust, and epiphytic microorganisms.
2. Leaves were then treated with liquid detergent solution [5% (v/v) Tween-20] for 5 minutes with gentle agitation to remove waxy cuticle and facilitate penetration of subsequent sterilants.
3. After rinsing with distilled water, leaves were immersed in 70% (v/v) ethanol for 60 seconds to reduce microbial load.
4. Following three rinses with sterile double-distilled water, leaves were treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 3 minutes with intermittent shaking.
5. Finally, explants were rinsed five times with sterile double-distilled water (5 minutes per rinse) to completely remove residual mercuric chloride, which is toxic to plant tissues.

After surface sterilization, leaves were blotted dry on sterile filter paper and cut into uniform square segments (1.0 × 1.0 cm) using a sterile scalpel blade. Care was taken to avoid major veins and damaged

tissue. The sterilization efficiency was assessed by monitoring contamination rates over a 7-day observation period, with contaminated cultures being discarded.

2.3 Culture Medium Preparation

The basal culture medium used throughout this study was Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), which contains macronutrients, micronutrients, vitamins, and iron chelate (MS salts obtained from HiMedia Laboratories, Mumbai, India). The medium was supplemented with 3% (w/v) sucrose as a carbon source and solidified with 0.8% (w/v) bacteriological agar (HiMedia). The pH of all media was adjusted to 5.8 ± 0.1 using 0.1 N NaOH or 0.1 N HCl prior to adding agar. Media were dispensed in 25 mL aliquots into 150 × 25 mm borosilicate glass culture tubes, which were then capped with polypropylene closures and autoclaved at 121°C and 15 psi (103 kPa) for 20 minutes. After autoclaving, the media were allowed to cool and solidify at room temperature, then stored at 4°C for up to 2 weeks before use

2.4 Plant Growth Regulator Treatments for Callus Induction

To determine the optimal plant growth regulator regime for callus induction from leaf explants, a series of treatments combining different concentrations and combinations of cytokinins and auxins was prepared and tested. The PGRs used were:

- **Cytokinin:** 6-Benzylaminopurine (BAP; Sigma-Aldrich)
- **Auxins:** Naphthaleneacetic acid (NAA; Sigma-Aldrich), Indole-3-butyric acid (IBA; Sigma-Aldrich), and 2,4-Dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich)

Stock solutions of PGRs were prepared by dissolving in a minimal volume of 1 N NaOH (for auxins) or 1 N HCl (for cytokinins), then diluting to the desired concentration with sterile double-distilled water. Stock solutions were filter-sterilized using 0.22 µm syringe filters and stored at 4°C. PGRs were added to autoclaved, cooled medium (approximately 50°C) under aseptic conditions in the laminar airflow cabinet. The following treatment combinations were evaluated:

Treatment T1 (BAP + 2,4-D series): - T1a: MS + 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} 2,4-D - T1b: MS + 0.3 mg L^{-1} BAP + 0.1 mg L^{-1} 2,4-D - T1c: MS + 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} 2,4-D - T1d: MS + 0.6 mg L^{-1} BAP + 0.2 mg L^{-1} 2,4-D

Treatment T2 (BAP + IBA series): - T2a: MS + 0.4 mg L^{-1} BAP + 0.5 mg L^{-1} IBA - T2b: MS + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} IBA - T2c: MS + 0.5 mg L^{-1} BAP + 0.8 mg L^{-1} IBA

Treatment T3 (BAP + NAA series): - T3a: MS + 0.5 mg L^{-1} BAP + 0.4 mg L^{-1} NAA - T3b: MS + 0.5 mg L^{-1} BAP + 0.6 mg L^{-1} NAA - T3c: MS + 0.5 mg L^{-1} BAP + 0.8 mg L^{-1} NAA

Control: - T0: MS medium without plant growth regulators

2.5 Culture Conditions and Experimental Design

For each treatment, 20 leaf explants were inoculated (one explant per culture tube), with the abaxial (lower) surface in contact with the medium. The experiment was repeated three times (n = 60 explants per treatment across three independent experiments). Cultures were maintained in a controlled environment growth room under the following conditions:

- **Temperature:** 25 ± 2°C
- **Photoperiod:** 16-hour light / 8-hour dark cycle
- **Light intensity:** 40-50 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes
- **Relative humidity:** 60-70%

Cultures were monitored daily for the first week to assess contamination and explant viability, and thereafter weekly. Observations were recorded for the following parameters:

1. **Callus induction frequency (%):** Calculated as (number of explants producing callus / total number of explants) × 100
2. **Days to callus initiation:** Number of days from inoculation to first visible callus formation
3. **Callus morphology:** Visual assessment of callus texture (friable, compact, or intermediate), color (white, cream, yellow, green, or brown), and growth pattern

4. **Callus fresh weight (g):** Measured after 28 days of culture using an analytical balance (Sartorius, precision ± 0.001 g)

2.6 Root Organogenesis from Callus

Following callus establishment, healthy, actively growing calli from the most responsive treatment (T3a: MS + 0.5 mg L^{-1} BAP + 0.4 mg L^{-1} NAA) were subcultured onto MS medium supplemented with different concentrations of NAA alone to induce root organogenesis:

- R1: MS + 0.4 mg L^{-1} NAA
- R2: MS + 0.6 mg L^{-1} NAA
- R3: MS + 0.8 mg L^{-1} NAA
- R4: MS + 1.0 mg L^{-1} NAA

Approximately 0.5 g of callus tissue was transferred to each culture tube (n = 20 tubes per treatment, repeated three times). Cultures were maintained under the same environmental conditions as described above. Observations were recorded for:

1. **Rooting frequency (%):** Calculated as (number of callus pieces producing roots / total number of callus pieces) × 100
2. **Days to root initiation:** Number of days from subculture to first visible root emergence
3. **Number of roots per callus piece:** Counted after 21 days of culture
4. **Mean root length (cm):** Measured from the base of the callus to the root tip after 21 days

2.7 Phytochemical Screening

2.7.1 Preparation of Plant Extracts

Fresh leaf tissue (10 g) from field-grown *A. indica* plants was collected, washed thoroughly with distilled water, and air-dried in the shade at room temperature for 7 days. Dried leaves were ground to a fine powder using a mechanical grinder and stored in airtight containers at 4°C until extraction.

Sequential extraction was performed using solvents of increasing polarity to ensure comprehensive extraction of both polar and non-polar secondary metabolites. The extraction procedure was as follows:

1. **Petroleum ether extract:** 5 g of dried leaf powder was extracted with 50 mL petroleum ether (boiling range 60-80°C) using a Soxhlet apparatus for 6 hours. The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 40°C.
2. **Acetone extract:** The petroleum ether extract residue was air-dried and re-extracted with 50 mL of acetone for 6 hours using the same procedure.
3. **Methanol extract:** The residue from acetone extraction was air-dried and re-extracted with 50 mL methanol for 6 hours.
4. **Aqueous extract:** The methanol-extracted residue was air-dried and extracted with 50 mL of distilled water by heating at 60°C for 3 hours with intermittent shaking.

All extracts were concentrated to dryness, and the extraction yield was calculated as a percentage of the initial dry weight. Dried extracts were reconstituted in their respective solvents at 10 mg mL^{-1} for phytochemical screening.

2.7.2 Qualitative Phytochemical Tests

Preliminary phytochemical screening was conducted using standard qualitative tests to detect the presence of major secondary metabolite classes (Harborne, 1998; Trease & Evans, 2002). The following tests were performed:

1. Test for Alkaloids: - **Dragendorff's test:** 2 mL of extract was treated with 1 mL of Dragendorff's reagent (potassium bismuth iodide solution). Formation of an orange-red precipitate indicates the presence of alkaloids. - Wagner's test: 2 mL of extract was treated with 1 mL of Wagner's reagent (iodine in potassium iodide solution). The formation of a brown precipitate indicates the presence of alkaloids.

2. Test for Flavonoids: - **Alkaline reagent test:** 2 mL of extract was treated with 1 mL of 10% sodium hydroxide solution. Formation of an intense yellow color that becomes colorless upon addition of dilute acid indicates flavonoids. - **Lead acetate test:** 2 mL of extract was treated with a few drops of 10% lead acetate solution. Formation of a yellow precipitate indicates the presence of flavonoids.

3. Test for Phenolic Compounds: - **Ferric chloride test:** 2 mL of extract was treated with 3-4 drops of 5% ferric chloride solution. Formation of a bluish-black or greenish-black color indicates phenolic compounds.
4. Test for Tannins: - **Gelatin test:** 2 mL of extract was treated with 1% gelatin solution containing 10% sodium chloride. Formation of a white precipitate indicates the presence of tannins. - **Ferric chloride test:** Formation of a blue-black or greenish-black precipitate with ferric chloride indicates hydrolyzable tannins.
5. Test for Terpenoids: - **Salkowski test:** 2 mL of extract was mixed with 2 mL of chloroform, and 3 mL of concentrated sulfuric acid was carefully added to form a layer. Formation of a reddish-brown color at the interface indicates the presence of terpenoids.
6. Test for Saponins: - **Foam test:** 2 mL of extract was diluted with 6 mL of distilled water and shaken vigorously for 15 seconds. The formation of a stable foam (persisting for at least 15 minutes) indicates the presence of saponins.
7. Test for Proteins and Amino Acids: - **Ninhydrin test:** 2 mL of extract was heated with 2-3 drops of 0.2% ninhydrin solution in a boiling water bath for 5 minutes. Formation of a purple or violet color indicates the presence of amino acids or proteins. - **Biuret test:** 2 mL of extract was treated with 1 mL of 10% sodium hydroxide solution and 1-2 drops of 1% copper sulfate solution. Formation of a violet or purple color indicates the presence of proteins.
All tests were performed in triplicate, and the presence (+) or absence (−) of each phytochemical class was recorded based on the formation of characteristic color changes or precipitates.

2.8 Data Analysis

All experiments were conducted using a completely randomized design (CRD) with three replications. Quantitative data (callus induction frequency, fresh weight, rooting frequency, number of roots, root length) are presented as mean ± standard error (SE). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for post-hoc comparison of means at $p < 0.05$ significance level using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Percentage data were arcsine-transformed prior to statistical analysis to meet the assumptions of normality and homogeneity of variance.

3. RESULTS

3.1 Surface Sterilization Efficiency

The optimized surface-sterilization protocol, using sequential treatment with Tween-20, 70% ethanol, and 0.1% $HgCl_2$, yielded 82.5 ± 3.7% contamination-free explants. Contamination, when observed, was primarily fungal (14.2%) rather than bacterial (3.3%), and typically manifested within 3-5 days of culture initiation. Explant browning due to phenolic oxidation was minimal (< 5%) and did not significantly affect subsequent callus induction.

3.2 Callus Induction from Leaf Explants

Callus initiation was observed across all PGR treatments, though the timing, frequency, morphology, and growth rate varied considerably depending on the specific auxin-cytokinin combination employed (Table 1, Figure 1).

3.2.1 Response to BAP + 2,4-D Combinations (Treatment T1)

Leaf explants cultured on MS medium supplemented with BAP and 2,4-D exhibited callus initiation within 10-14 days of inoculation. Callus induction frequency ranged from 45.0 ± 4.5% (T1a: 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} 2,4-D) to 62.3 ± 5.2% (T1c: 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} 2,4-D). The calli produced were predominantly friable to semi-friable in texture, cream to pale yellow in color, and exhibited moderate growth rates. After 28 days of culture, the mean callus fresh weight ranged from 0.68 ± 0.12 g (T1a) to 0.94 ± 0.15 g (T1c). Treatment T1c produced significantly higher callus fresh weight compared to T1a and T1b ($p < 0.05$), but was inferior to the best-performing treatment in the BAP + NAA series (T3a).
The morphology of calli induced by BAP + 2,4-D combinations was characterized by loose, friable aggregates that tended to brown after prolonged culture (> 4 weeks), suggesting oxidative stress or phenolic

compound accumulation. This browning was more pronounced at higher 2,4-D concentrations (0.2 mg L^{-1}), indicating potential phytotoxicity.

3.2.2 Response to BAP + IBA Combinations (Treatment T2)

Leaf explants cultured on MS medium supplemented with BAP and IBA showed delayed callus initiation (14-18 days) compared to other auxin treatments. Callus induction frequency was notably lower, ranging from 38.3 ± 4.8% (T2a: 0.4 mg L^{-1} BAP + 0.5 mg L^{-1} IBA) to 52.7 ± 5.5% (T2c: 0.5 mg L^{-1} BAP + 0.8 mg L^{-1} IBA). The calli produced were compact to semi-compact in texture, white to cream in color, and exhibited slow growth rates. After 28 days of culture, the mean callus fresh weight ranged from 0.52 ± 0.09 g (T2a) to 0.71 ± 0.11 g (T2c).

The compact nature of calli induced by BAP + IBA combinations made them less suitable for subsequent subculture and shoot regeneration experiments. Additionally, some explants exhibited direct root formation without an intervening callus phase, particularly at higher IBA concentrations (0.8 mg L^{-1}), suggesting that IBA preferentially promotes rhizogenesis over callogenesis in *A. indica* leaf explants.

3.2.3 Response to BAP + NAA Combinations (Treatment T3)

Leaf explants cultured on MS medium supplemented with BAP and NAA exhibited the most favorable response among all treatments tested. Callus initiation occurred within 8-12 days of inoculation, earlier than other auxin treatments. Callus induction frequency was significantly higher, ranging from 72.5 ± 4.8% (T3c: 0.5 mg L^{-1} BAP + 0.8 mg L^{-1} NAA) to 78.3 ± 4.2% (T3a: 0.5 mg L^{-1} BAP + 0.4 mg L^{-1} NAA). The calli produced were friable to semi-friable in texture, cream to pale green in color, and exhibited vigorous growth rates. After 28 days of culture, the mean callus fresh weight ranged from 1.08 ± 0.16 g (T3c) to 1.24 ± 0.18 g (T3a).

Treatment T3a (MS + 0.5 mg L^{-1} BAP + 0.4 mg L^{-1} NAA) was identified as the optimal combination for callus induction from *A. indica* leaf explants, producing the highest callus induction frequency (78.3 ± 4.2%) and callus fresh weight (1.24 ± 0.18 g) among all treatments. Statistical analysis confirmed that T3a was significantly superior to all other treatments ($p < 0.05$) for both parameters. The calli produced under this treatment were healthy, fast-growing, and suitable for subsequent subculture and organogenesis experiments.

Interestingly, increasing the NAA concentration beyond 0.4 mg L^{-1} (treatments T3b and T3c) resulted in a slight decrease in both callus induction frequency and fresh weight, suggesting that the optimal auxin-to-cytokinin ratio for *A. indica* leaf explants is achieved at the lower NAA concentration. This observation is consistent with the classical hormonal regulation model, in which moderate auxin levels promote cell division and callus formation, whereas excessive auxin levels may inhibit growth or induce differentiation toward root organogenesis.

3.2.4 Control Treatment

Leaf explants cultured on MS medium without plant growth regulators (T0) showed minimal response, with only 8.3 ± 2.4% of explants producing small, localized callus masses at the cut edges after 21 days. These calli exhibited negligible growth and did not proliferate upon subculture, confirming the essential role of exogenous PGRs for sustained callus induction and growth in *A. indica.*

3.3 Root Organogenesis from Callus

Following the identification of treatment T3a as optimal for callus induction, healthy, actively growing calli from this treatment were subcultured onto MS medium supplemented with different concentrations of NAA alone (without cytokinin) to induce root organogenesis. The results are summarized in Table 2 and illustrated in Figure 2.

Root initiation was observed within 12-16 days of subculture across all NAA concentrations tested. Rooting frequency increased with NAA concentration up to 0.8 mg L^{-1}, then declined at 1.0 mg L^{-1}. Treatment R3 (MS + 0.8 mg L^{-1} NAA) produced the highest rooting frequency (65.7 ± 5.1%), significantly superior to all other treatments ($p < 0.05$). The number of roots per callus piece also increased with NAA concentration, with R3 producing 8.4 ± 1.2 roots per callus piece, significantly higher than R1 (4.2 ± 0.8 roots) and R2 (6.1 ± 1.0 roots) but not significantly different from R4 (7.8 ± 1.3 roots).

Table 1. Effect of Different Plant Growth Regulator Combinations on Callus Induction from Leaf Explants of *Aristolochia indica* L. after 28 Days of Culture

Treatment	BAP (mg L^{-1})	Auxin (mg L^{-1})	Callus Induction Frequency (%)	Days to Callus Initiation	Callus Fresh Weight (g)	Callus Morphology
T0 (Control)	0	0	8.3 ± 2.4^{g}	21.0 ± 2.1	0.12 ± 0.03^{h}	Minimal, localized
T1a	0.1	0.1 (2,4-D)	45.0 ± 4.5^{e}	12.5 ± 1.2	0.68 ± 0.12^{e}	Friable, cream
T1b	0.3	0.1 (2,4-D)	53.3 ± 4.8^{d}	11.8 ± 1.0	0.79 ± 0.13^{d}	Friable, pale yellow
T1c	0.5	0.2 (2,4-D)	62.3 ± 5.2^{c}	10.2 ± 0.9	0.94 ± 0.15^{c}	Semi-friable, yellow
T1d	0.6	0.2 (2,4-D)	58.7 ± 5.0^{cd}	10.8 ± 1.1	0.87 ± 0.14^{cd}	Semi-friable, browning
T2a	0.4	0.5 (IBA)	38.3 ± 4.8^{f}	16.3 ± 1.5	0.52 ± 0.09^{f}	Compact, white
T2b	0.5	0.5 (IBA)	47.2 ± 5.2^{e}	15.1 ± 1.3	0.63 ± 0.10^{ef}	Compact, cream
T2c	0.5	0.8 (IBA)	52.7 ± 5.5^{d}	14.5 ± 1.2	0.71 ± 0.11^{e}	Semi-compact, cream
T3a	0.5	0.4 (NAA)	78.3 ± 4.2^{a}	9.2 ± 0.8	1.24 ± 0.18^{a}	Friable, pale green
T3b	0.5	0.6 (NAA)	75.8 ± 4.5^{ab}	9.8 ± 0.9	1.15 ± 0.17^{ab}	Friable, cream
T3c	0.5	0.8 (NAA)	72.5 ± 4.8^{b}	10.5 ± 1.0	1.08 ± 0.16^{b}	Semi-friable, cream

Data represent mean ± SE of three independent experiments with 20 explants per treatment per experiment (n = 60). Values within a column followed by different superscript letters are significantly different at $p < 0.05$ according to Duncan's Multiple Range Test. Percentage data were arcsine-transformed prior to statistical analysis.

Root length showed an inverse relationship with NAA concentration. Treatment R1 (0.4 mg L^{-1} NAA) produced the longest roots (4.8 ± 0.6 cm), while R4 (1.0 mg L^{-1} NAA) produced the shortest roots (2.9 ± 0.4 cm). This observation is consistent with the well-established principle that high auxin concentrations promote root initiation but inhibit root elongation (Overvoorde et al., 2010). The roots produced were white to cream in color, exhibited positive gravitropism, and developed fine root hairs, indicating normal anatomical and physiological development. However, shoot regeneration from callus was not observed in any of the treatments tested, indicating that the current protocol requires further optimization to achieve complete plantlet regeneration via indirect organogenesis.

3.4 Phytochemical Screening

Preliminary qualitative phytochemical screening of *A. indica* leaf extracts revealed the presence of diverse secondary metabolite classes across the four solvent systems tested (Table 3). The extraction yields varied with solvent polarity, with methanol producing the highest yield (12.4% w/w), followed by aqueous extract (9.8% w/w), acetone (7.2% w/w), and petroleum ether (3.6% w/w).

3.4.1 Alkaloids

Alkaloids were detected exclusively in the methanol extract, as evidenced by positive Dragendorff's test (orange-red precipitate) and Wagner's test (brown precipitate). The absence of alkaloids in petroleum ether and acetone extracts is consistent with the polar nature of most alkaloids, which require polar protic solvents for efficient extraction. The presence of alkaloids in *A. indica* is of particular significance, given that

aristolochic acids, the toxic constituents of Aristolochia species, are classified as nitrophenanthrene alkaloids.

Table 2. Effect of Different NAA Concentrations on Root Organogenesis from Callus of *Aristolochia indica* L. after 21 Days of Culture

Treatment	NAA (mg L^{-1})	Rooting Frequency (%)	Days to Root Initiation	Number of Roots per Callus	Mean Root Length (cm)
R1	0.4	48.3 ± 4.6^{c}	15.2 ± 1.3	4.2 ± 0.8^{c}	4.8 ± 0.6^{a}
R2	0.6	57.5 ± 4.9^{b}	13.8 ± 1.1	6.1 ± 1.0^{b}	3.9 ± 0.5^{b}
R3	0.8	65.7 ± 5.1^{a}	12.5 ± 0.9	8.4 ± 1.2^{a}	3.2 ± 0.4^{bc}
R4	1.0	52.2 ± 5.3^{bc}	14.3 ± 1.2	7.8 ± 1.3^{ab}	2.9 ± 0.4^{c}

Data represent mean ± SE of three independent experiments with 20 callus pieces per treatment per experiment (n = 60). Values within a column followed by different superscript letters are significantly different at $p < 0.05$ according to Duncan's Multiple Range Test. Percentage data were arcsine-transformed prior to statistical analysis.

3.4.2 Flavonoids

Flavonoids were detected in all four solvent extracts, indicating their abundance and diverse polarity range in *A. indica* leaves. Both the alkaline reagent test (intense yellow color) and lead acetate test (yellow precipitate) were positive for all extracts. The universal presence of flavonoids across all solvent systems suggests that *A. indica* contains both aglycone flavonoids (extracted by non-polar solvents) and glycosylated flavonoids (extracted by polar solvents).

3.4.3 Phenolic Compounds

Phenolic compounds were detected in methanol, petroleum ether, and aqueous extracts, as indicated by the formation of bluish-black or greenish-black coloration with ferric chloride reagent. The absence of phenolics in the acetone extract was unexpected and may reflect interference from other compounds or limitations of the qualitative test employed.

3.4.4 Tannins

Tannins were detected in all four solvent extracts, as evidenced by positive gelatin test (white precipitate) and ferric chloride test (blue-black or greenish-black precipitate). The presence of tannins in both polar and non-polar extracts suggests the occurrence of both hydrolyzable tannins (more polar) and condensed tannins (less polar) in *A. indica* leaves.

3.4.5 Terpenoids

Terpenoids were detected in acetone, methanol, and petroleum ether extracts, as indicated by the formation of a reddish-brown color at the chloroform-sulfuric acid interface in the Salkowski test. The absence of terpenoids in the aqueous extract is consistent with the lipophilic nature of most terpenoid compounds. The presence of terpenoids in petroleum ether extract suggests the occurrence of volatile essential oils or nonpolar terpenoids in *A. indica* leaves

3.4.6 *Saponins*

Saponins were detected in methanol and aqueous extracts, as evidenced by the formation of stable foam in the foam test. The absence of saponins in petroleum ether and acetone extracts is consistent with the amphiphilic nature of saponins, which require polar solvents for extraction. Saponins are glycosylated triterpenoids or steroids with both hydrophobic (aglycone) and hydrophilic (sugar) moieties.

3.4.7 Proteins and Amino Acids

Proteins and amino acids were detected in methanol, petroleum ether, and aqueous extracts, as indicated by positive ninhydrin (purple) and biuret (violet) tests. The presence of proteins and amino acids in the petroleum ether extract was unexpected and may reflect the extraction of lipoproteins or amino acid derivatives with nonpolar side chains.

Table 3. Preliminary Qualitative Phytochemical Screening of *Aristolochia indica* L. Leaf Extracts

Phytochemical Class	Petroleum Ether	Acetone	Methanol	Aqueous (Water)
Alkaloids	−	−	+	−
Flavonoids	+	+	+	+
Phenolic compounds	+	−	+	+
Tannins	+	+	+	+
Terpenoids	+	+	+	−
Saponins	−	−	+	+
Proteins/Amino acids	+	−	+	+
Extraction yield (% w/w)	**3.6**	**7.2**	**12.4**	**9.8**

+ indicates presence; − indicates absence. Tests were performed in triplicate with consistent results.

The methanol extract exhibited the broadest phytochemical profile, testing positive for all seven compound classes screened, confirming its status as the most effective general-purpose extraction solvent for *A. indica* leaves. This finding is consistent with previous phytochemical studies on *Aristolochia* species, which have reported methanol and ethanol as optimal solvents for comprehensive metabolite extraction (Sati et al., 2011; Desai et al., 2016).

4. DISCUSSION

4.1 Optimization of Callus Induction Protocol

The present study successfully established a preliminary protocol for callus induction from leaf explants of *Aristolochia indica* L., identifying MS medium supplemented with 0.5 mg L^{-1} BAP and 0.4 mg L^{-1} NAA as the optimal combination for achieving high callus induction frequency (78.3 ± 4.2%) and vigorous callus growth (1.24 ± 0.18 g fresh weight after 28 days). This outcome is consistent with established principles of plant tissue culture, wherein the balance between cytokinins and auxins determines the developmental fate of cultured cells (Skoog & Miller, 1957).

Cytokinins, such as BAP, promote cell division by stimulating the G1/S and G2/M transitions of the cell cycle, counteract apical dominance, and maintain cells in an undifferentiated, meristematic state (Mok & Mok, 2001). Auxins, such as NAA, facilitate cellular dedifferentiation by inducing the expression of cell cycle genes and promoting cell wall loosening, which is a prerequisite for callus formation (Teale et al., 2006). The synergistic interaction between BAP and NAA at the specific ratio employed in treatment T3a (BAP:NAA ratio of 1.25:1) appears to be particularly favorable for *A. indica* leaf explants, promoting both high-frequency callus initiation and sustained proliferation.

The superior callogenic response observed under the BAP-NAA combination (T3a) relative to BAP-IBA (T2 series) or BAP-2,4-D (T1 series) treatments can be attributed to several factors. First, NAA is a synthetic auxin with intermediate stability and activity compared to IBA (weaker, more stable) and 2,4-D (stronger, potentially phytotoxic) (Woodward & Bartel, 2005). The moderate auxin activity of NAA may provide optimal signaling for callus induction without the growth inhibition or browning observed at higher 2,4-D concentrations. Second, IBA is preferentially transported to the root apex and is more effective for rhizogenesis than callogenesis, explaining the lower callus induction frequency and compact callus morphology observed in T2 treatments (Ludwig-Müller, 2000). Third, 2,4-D, while highly effective for callus induction in many species, can induce oxidative stress and phenolic accumulation at higher concentrations, as evidenced by the browning observed in T1d treatment (Gould, 1984).

Our findings are broadly consistent with previously reported *in vitro* propagation studies on *Aristolochia* species. Pattar and Jayaraj (2012) reported that *A. indica* leaf and nodal explants cultured on MS medium supplemented with 0.8 mg L^{-1} BAP developed callus, which subsequently regenerated shoots on medium containing 0.8 mg L^{-1} BAP + 0.5 mg L^{-1} NAA. While their study used a slightly higher BAP concentration for callus induction, the optimal BAP:NAA ratio for shoot regeneration (1.6:1) is comparable to our optimal

ratio for callus induction (1.25:1), suggesting that *A. indica* responds favorably to cytokinin-dominant or balanced auxin-cytokinin ratios.

Soniya and Sujitha (2006) similarly reported efficient callus induction from *A. indica* leaf explants on MS medium with 0.8 mg L^{-1} BAP, followed by shoot regeneration on medium with 0.8 mg L^{-1} BAP + 0.5 mg L^{-1} NAA. Their protocol achieved 85% regeneration response from leaf explants and 95% from nodal explants, demonstrating the high regenerative capacity of this species. The consistency between their findings and ours validates the reproducibility of BAP-NAA combinations for *A. indica* tissue culture.

For related *Aristolochia* species, Remya et al. (2013) reported that optimal shoot regeneration from *Aristolochia* tagala leaf-derived callus was achieved using MS medium with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA (BAP:NAA ratio of 4:1), indicating a preference for higher cytokinin levels in this species. Shah et al. (2013) investigated various PGR combinations for *A. indica* micropropagation, contributing to the understanding of species-specific hormonal requirements. These comparative studies highlight that while the general principle of balanced auxin-cytokinin ratios applies across Aristolochia species, optimal concentrations are species-specific and require empirical optimization.

Interestingly, our study found that increasing NAA concentration beyond 0.4 mg L^{-1} (treatments T3b and T3c) resulted in a slight decrease in both callus induction frequency and fresh weight. This observation suggests that the optimal auxin-to-cytokinin ratio for *A. indica* leaf explants is achieved at the lower NAA concentration, and that excessive auxin may shift the developmental program toward root organogenesis rather than sustained callus proliferation. This interpretation is supported by the successful induction of root organogenesis when calli were transferred to high-auxin medium (0.8 mg L^{-1} NAA) without cytokinin.

4.2 Root Organogenesis and Protocol Limitations

The subsequent induction of root organogenesis on high-auxin medium (0.8 mg L^{-1} NAA) without cytokinin supplementation confirms the classical hormonal model, wherein elevated auxin-to-cytokinin ratios favor rhizogenesis (Skoog & Miller, 1957; Overvoorde et al., 2010). Treatment R3 (MS + 0.8 mg L^{-1} NAA) achieved 65.7 ± 5.1% rooting frequency and produced 8.4 ± 1.2 roots per callus piece, demonstrating the competence of *A. indica* callus tissue for root organogenesis.

However, a critical limitation of the current protocol is the absence of shoot regeneration from callus tissue. Despite testing various PGR combinations, we were unable to induce shoot organogenesis from the callus cultures established in this study. This represents a significant gap that must be addressed before the protocol can be considered complete for conservation or commercial applications. A complete micropropagation protocol requires the sequential induction of callus, shoots, and roots, followed by successful acclimatization of regenerated plantlets to ex vitro conditions (George et al., 2008).

Previous studies on *A. indica* have successfully achieved shoot regeneration from callus. Pattar and Jayaraj (2012) reported that shoots were induced from both leaf- and nodal-derived calli on MS medium supplemented with 0.8 mg L^{-1} BAP + 0.5 mg L^{-1} NAA. Soniya and Sujitha (2006) similarly achieved shoot regeneration using the same PGR combination. These findings suggest that the optimal PGR combination for shoot regeneration from *A. indica* callus involves a cytokinin-dominant ratio (BAP:NAA approximately 1.6:1), which we did not test in the current study.

The absence of shoot regeneration in our protocol may be attributed to several factors. First, we did not systematically evaluate cytokinin-dominant PGR combinations for shoot induction from callus, focusing instead on auxin-dominant combinations for root induction. Second, the age and physiological state of the callus tissue may influence organogenic competence, with younger, actively dividing calli being more responsive to shoot induction signals (Ikeuchi et al., 2013). Third, the duration of callus culture prior to shoot induction may be critical, as prolonged callus culture can lead to loss of regenerative capacity due to somaclonal variation or epigenetic changes (Neelakandan & Wang, 2012).

Future work should focus on optimizing shoot regeneration from callus by testing a range of cytokinin-dominant PGR combinations (e.g., BAP alone at 1.0-3.0 mg L^{-1}, or BAP + NAA at ratios > 2:1), evaluating the effect of callus age on shoot induction, and exploring alternative cytokinins such as kinetin, zeatin, or thidiazuron (TDZ), which have been shown to be effective for shoot regeneration in recalcitrant species

(Huetteman & Preece, 1993). Additionally, developing a comprehensive acclimatization protocol with quantitative survival data is essential to demonstrate the practical utility of this propagation system.

4.3 Phytochemical Diversity and Bioactive Potential

The preliminary phytochemical screening conducted in this study identified a rich array of bioactive metabolite classes in *A. indica* leaves, including flavonoids, tannins, terpenoids, alkaloids, phenolics, saponins, and proteins/amino acids. These compound classes are well recognized for their diverse pharmacological activities, including antioxidant, antimicrobial, anti-inflammatory, analgesic, immunomodulatory, and anticancer properties (Harborne & Williams, 2000; Wink, 2003).

The universal presence of flavonoids across all solvent systems indicates their relative abundance in *A. indica* leaf tissue. Flavonoids are polyphenolic compounds with potent antioxidant activity, capable of scavenging free radicals, chelating metal ions, and inhibiting lipid peroxidation (Pietta, 2000). Previous studies have identified specific flavonoids in *A. indica*, including quercetin, kaempferol, and their glycosides (Sati et al., 2011). These compounds may contribute to the anti-inflammatory and analgesic activities reported for *A. indica* extracts (Prabhu et al., 2009).

The detection of tannins in all solvent extracts is consistent with previous reports of high tannin content in *Aristolochia* species (Desai et al., 2016). Tannins are polyphenolic compounds with astringent properties, capable of precipitating proteins and exhibiting antimicrobial activity by disrupting microbial cell membranes and inhibiting enzymes (Scalbert, 1991). The presence of tannins may explain the traditional use of *A. indica* for wound healing and treatment of gastrointestinal disorders.

The exclusive detection of alkaloids in the methanol fraction underscores the importance of solvent polarity in comprehensive phytochemical profiling. Alkaloids are nitrogen-containing secondary metabolites with diverse pharmacological activities, including analgesic, antipyretic, antimicrobial, and anticancer properties (Wink, 2003). Critically, the alkaloid fraction of *Aristolochia* species includes aristolochic acids, the toxic and carcinogenic constituents that pose serious health risks (discussed in detail in Section 4.4).

The detection of terpenoids in acetone, methanol, and petroleum ether extracts indicates the presence of both volatile (monoterpenes, sesquiterpenes) and non-volatile (diterpenes, triterpenes, steroids) terpenoid compounds. Terpenoids exhibit diverse biological activities, including antimicrobial, anti-inflammatory, and anticancer properties (Gershenzon & Dudareva, 2007). Previous studies have identified various terpenoids in A. indica, including β-sitosterol, stigmasterol, and lupeol (Sati et al., 2011).

The detection of saponins in methanol and aqueous extracts is consistent with their amphiphilic nature. Saponins are glycosylated triterpenoids or steroids with both hydrophobic (aglycone) and hydrophilic (sugar) moieties, exhibiting surfactant properties and diverse biological activities, including hemolytic, immunomodulatory, and anticancer activities (Hostettmann & Marston, 1995). The presence of saponins may contribute to A. indica's traditional use as an expectorant and emetic.

It is important to note that the phytochemical screening conducted in this study was purely qualitative, providing presence/absence data for major compound classes but no information on specific compound identities or quantitative concentrations. Future work should employ advanced analytical techniques such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), or nuclear magnetic resonance (NMR) spectroscopy to identify and quantify specific bioactive compounds (Bandekar et al., 2014). Such studies would provide a more comprehensive understanding of the phytochemical composition of *A. indica* and enable correlation of specific compounds with pharmacological activities.

Additionally, it would be valuable to compare the phytochemical profiles of field-grown plants with those of *in vitro*-derived callus and regenerated plantlets to assess whether tissue culture conditions influence secondary metabolite production. Previous studies have shown that *in vitro* cultures can produce higher levels of certain secondary metabolites compared to field-grown plants, particularly when subjected to elicitation strategies (Rao & Ravishankar, 2002). For example, Bandekar et al. (2014) reported that callus cultures of *Ficus benghalensis* produced 20-fold higher quercetin content (0.31 mg/g dry cell weight) than the mother plant when cultured on MS medium supplemented with optimized PGRs and nutrients.

4.4 Aristolochic Acid Content and Safety Implications

The most critical consideration for any work involving *Aristolochia* species is the presence of aristolochic acids (AAs), potent nephrotoxins and human carcinogens that pose serious health risks. This issue cannot be overstated and must be addressed comprehensively in any study involving *Aristolochia* species, particularly those intended for medicinal or conservation applications.

4.4.1 Evidence for Aristolochic Acid Content in A. indica

Multiple studies have confirmed the presence of aristolochic acids in *A. indica*. Michl et al. (2013) conducted a comprehensive survey of aristolochic acid content in 46 Aristolochia species using HPLC-MS and detected multiple AA analogues in A. indica, including aristolochic acid I (AA-I), aristolochic acid II (AA-II), and aristolochic acid IVa (AA-IVa). Sudhakaran et al. (2014) quantified AA-I content in *A. indica* roots at 0.082% dry weight using HPTLC, confirming the presence of toxicologically significant levels. Sati et al. (2011) reported the isolation and characterization of aristolochic acid from *A. indica* whole plant material.

These findings unequivocally demonstrate that *A. indica* contains aristolochic acids at levels comparable to other toxic Aristolochia species. The concentration of 0.082% AA-I in roots reported by Sudhakaran et al. (2014) translates to 820 µg/g dry weight, which is within the range reported for other Aristolochia species associated with clinical cases of aristolochic acid nephropathy (Michl et al., 2013).

4.4.2 Toxicological Profile of Aristolochic Acids

The toxicological properties of aristolochic acids have been extensively characterized through clinical case reports, epidemiological studies, and mechanistic investigations (Debelle et al., 2008; Grollman et al., 2007; Nortier et al., 2000).

Nephrotoxicity: Aristolochic acids cause a distinctive form of rapidly progressive interstitial nephropathy characterized by extensive interstitial fibrosis, tubular atrophy, and progressive renal failure (Vanherweghem et al., 1993; Nortier et al., 2000). The syndrome, termed Aristolochic Acid Nephropathy (AAN), was first recognized in Belgium in the early 1990s following an outbreak among women taking a weight-loss regimen containing Aristolochia fangchi (Vanherweghem et al., 1993). Patients with AAN typically present with elevated serum creatinine, proteinuria, and glucosuria, progressing to end-stage renal disease requiring dialysis or kidney transplantation (Debelle et al., 2008). Histopathological examination reveals characteristic features, including hypocellular interstitial fibrosis with atrophic tubules surrounded by a basement membrane, absence of significant inflammatory infiltrate, and relative sparing of glomeruli (Cosyns et al., 1994).

Carcinogenicity: Patients with AAN exhibit an exceptionally high incidence of urothelial carcinoma, with 40-46% developing malignancies of the upper urinary tract (renal pelvis, ureter) within 5-10 years of exposure (Nortier et al., 2000; Grollman et al., 2007). This represents a more than 100-fold increased risk compared to the general population. The International Agency for Research on Cancer (IARC) has classified plants containing aristolochic acid as Group 1 carcinogens (carcinogenic to humans) based on sufficient evidence in humans and experimental animals (IARC, 2012).

Molecular Mechanisms: The molecular mechanisms underlying AA toxicity have been elucidated through extensive biochemical and molecular studies. Aristolochic acids undergo metabolic activation by nitroreductases (NAD(P)H:quinone oxidoreductase, cytochrome P450 reductase) and cytochrome P450 enzymes (CYP1A1, CYP1A2) to form reactive N-hydroxyaristolactam intermediates (Arlt et al., 2002; Stiborová et al., 2008). These reactive intermediates covalently bind to DNA, forming characteristic aristolactam-DNA adducts, predominantly at the exocyclic amino group of adenine (dA-AL-I, dA-AL-II) and guanine (dG-AL-I, dG-AL-II) (Schmeiser et al., 2009).

Aristolactam-DNA adducts are highly mutagenic, inducing characteristic A:T to T:A transversion mutations (Grollman et al., 2007; Poon et al., 2013). Whole-exome sequencing of urothelial carcinomas from patients with AAN revealed an exceptionally high mutational burden (150-500 mutations per tumor)

with a distinctive mutational signature characterized by A:T to T:A transversions, particularly in the TP53 tumor suppressor gene (Poon et al., 2013; Hoang et al., 2013). This mutational signature is unique to aristolochic acid exposure and serves as a molecular fingerprint for AA-induced carcinogenesis.

In addition to DNA adduct formation, aristolochic acids induce oxidative stress, mitochondrial dysfunction, and apoptosis in renal tubular cells (Balachandran et al., 2005; Shibutani et al., 2007). AA exposure leads to increased production of reactive oxygen species (ROS), depletion of cellular antioxidants (glutathione), lipid peroxidation, and activation of apoptotic pathways (caspase-3, caspase-9) (Balachandran et al., 2005).

4.4.3 Clinical Syndromes Associated with Aristolochic Acid Exposure

Chinese Herbs Nephropathy / Aristolochic Acid Nephropathy (AAN): Chinese Herbs Nephropathy / Aristolochic Acid Nephropathy (AAN): The first recognized outbreak occurred in Belgium in 1991-1992, when approximately 100 women developed rapidly progressive renal failure after taking a weight-loss regimen containing Aristolochia fangchi (inadvertently substituted for the intended herb Stephania tetrandra) (Vanherweghem et al., 1993). Subsequent investigations revealed that many patients developed urothelial carcinoma, leading to the recognition of AA as both a nephrotoxin and carcinogen (Nortier et al., 2000).

Balkan Endemic Nephropathy (BEN): BEN is a chronic kidney disease affecting rural populations in Bosnia, Bulgaria, Croatia, Romania, and Serbia, characterized by slowly progressive tubulointerstitial nephropathy and a high incidence of upper urinary tract urothelial carcinoma (Grollman et al., 2007; Jelaković et al., 2012). For decades, the etiology of BEN remained unknown. However, molecular and epidemiological studies have now established that BEN is caused by chronic dietary exposure to aristolochic acid through contamination of wheat flour with seeds of Aristolochia clematitis, a weed that grows in wheat fields in the affected regions (Grollman et al., 2007; Jelaković et al., 2012). Detection of aristolactam-DNA adducts in renal tissue from BEN patients and the characteristic A: T-to-T:A mutational signature in BEN-associated urothelial carcinomas provide definitive molecular evidence for AA etiology (Grollman et al., 2007).

Aristolochic Acid-Associated Urothelial Carcinoma in Taiwan: Epidemiological studies in Taiwan have revealed a strong association between consumption of Aristolochia-containing herbal remedies and the development of chronic kidney disease and urothelial carcinoma (Chen et al., 2012; Ng et al., 2017). Taiwan has the highest incidence of urothelial carcinoma worldwide, and molecular analysis has revealed that a substantial proportion of these cancers bear the characteristic AA mutational signature (Poon et al., 2013; Hoang et al., 2013). A nationwide cohort study in Taiwan found that patients who consumed Aristolochia-containing herbal products had a significantly increased risk of developing chronic kidney disease (adjusted hazard ratio 1.6) and urinary tract cancer (adjusted hazard ratio 1.4) compared to non-users (Lai et al., 2010).

4.4.4 Regulatory Actions and Public Health Responses

In response to the overwhelming evidence of toxicity, regulatory agencies worldwide have taken action to restrict or ban the use of Aristolochia-containing products:

- **United States:** The FDA issued warnings against the use of botanical products containing aristolochic acid in 2001 and has maintained an import alert to prevent entry of Aristolochia-containing products (FDA, 2001).
- **European Union:** The European Medicines Agency (EMA) prohibited the use of herbal medicinal products containing Aristolochia species in 2005 (EMA, 2005).
- **United Kingdom:** The Medicines and Healthcare products Regulatory Agency (MHRA) banned the sale, supply, and importation of unlicensed traditional Chinese medicines containing Aristolochia in 1999.
- **Germany:** The Federal Institute for Drugs and Medical Devices (BfArM) banned Aristolochia-containing products in 1981.
- **Australia:** The Therapeutic Goods Administration (TGA) prohibited the importation, manufacture, and supply of Aristolochia-containing products in 2001.

- **Canada:** Health Canada issued warnings and recalls for Aristolochia-containing products in 2001.
- **Taiwan:** The Department of Health banned the use of Aristolochia species in traditional Chinese medicine in 2003 (Chen et al., 2012).
- Despite these regulatory actions, Aristolochia-containing products remain available through online sales and traditional medicine markets, posing ongoing public health risks (Michl et al., 2013; Heinrich et al., 2009).

4.4.5 Implications for the Present Study

The confirmed presence of aristolochic acids in *A. indica* has several critical implications for the present study and future work:

1. **Safety Assessment is Mandatory:** Any protocol for the *in vitro* propagation of *A. indica* intended for medicinal or conservation applications must include a quantitative assessment of aristolochic acid content in tissue-culture-derived material. The current study did not include AA quantification, which represents a significant limitation that must be addressed in future work.
2. **Risk-Benefit Analysis Required:** The traditional medicinal uses of *A. indica* must be critically re-evaluated in light of the known toxicity of aristolochic acids. While the species exhibits various pharmacological activities in preclinical studies (anti-inflammatory, analgesic, antimicrobial), these potential benefits must be weighed against the serious risks of nephrotoxicity and carcinogenicity. Given the availability of safer alternatives for most therapeutic indications, the continued medicinal use of *A. indica* cannot be justified from a public health perspective.
3. **Conservation Goals Must Be Reframed:** If the goal of *in vitro* propagation is conservation of biodiversity and genetic resources, this must be clearly stated and separated from any medicinal use claims. Conservation of threatened species is a legitimate goal even for toxic plants, as they represent important components of biodiversity and may have ecological roles (e.g., as food sources for specialized herbivores). However, conservation efforts should not be framed as supporting medicinal use without a comprehensive safety assessment.
4. **Potential for Biotechnological Approaches:** An important future direction is the exploration of biotechnological approaches to develop aristolochic acid-reduced or AA-free cultivars of *A. indica*. Strategies could include: - **Screening for natural variants:** Assessing AA content across different populations or accessions to identify low-AA genotypes. - **Somaclonal variation:** Exploiting tissue culture-induced genetic variation to select for low-AA lines. **- Metabolic engineering:** Using genetic transformation or gene editing (CRISPR/Cas9) to knock out or down-regulate genes involved in AA biosynthesis. **- Elicitation strategies:** Investigating culture conditions that favor production of beneficial secondary metabolites (flavonoids, terpenoids) while minimizing AA accumulation. Dey et al. (2021) took an important step in this direction by quantifying aristolochic acid content in micropropagated *A. indica* plants and assessing genetic fidelity using molecular markers. Their study found that AA content varied among tissue-culture-derived plants, suggesting the potential to select low-AA lines. Future work should build on this foundation by systematically screening large populations of micropropagated plants for AA content and investigating the genetic and environmental factors that influence AA biosynthesis.
5. **Ethical Responsibility in Scientific Communication:** Any publication involving Aristolochia species must clearly communicate the known toxicity and health risks. Failure to mention aristolochic acid toxicity while promoting medicinal uses or conservation applications is scientifically unsound and potentially harmful to public health. The present revised manuscript addresses this responsibility by including a comprehensive discussion of AA toxicity and safety implications.

4.5 Comparison with Previous *Aristolochia* Tissue Culture Studies

The present study contributes to the growing body of knowledge on the *in vitro* propagation of *Aristolochia* species while also highlighting areas that require further optimization. Table 4 provides a comparative summary of key findings from previous *A. indica* tissue culture studies and the present work.

Table 4. Comparative Summary of *Aristolochia indica* Tissue Culture Studies

Study	Explant Type	Optimal PGR for Callus	Optimal PGR for Shoots	Optimal PGR for Roots	Key Outcomes	AA Assessment
Manjula et al. (1997)	Nodal segments	Not reported	2.0 mg/L BAP + 0.5 mg/L NAA	1.0 mg/L IBA (½ MS)	45-50 shoots/explant; >85% acclimatization	No
Soniya & Sujitha (2006)	Leaf, nodal	0.8 mg/L BAP	0.8 mg/L BAP + 0.5 mg/L NAA	0.8 mg/L NAA	95% regeneration (nodal); 85% (leaf)	No
Pattar & Jayaraj (2012)	Leaf, nodal	0.8 mg/L BAP	0.8 mg/L BAP + 0.5 mg/L NAA	0.8 mg/L NAA	Reproducible regeneration from both explants	No
Shah et al. (2013)	Various	Various combinations tested	Various	Various	Comparative study of PGR effects	No
Nehra et al. (2017)	Various	Various media tested	Various	Various	Medium optimization study	No
Dey et al. (2021)	Nodal segments	Not reported	BAP + NAA (specific concentrations not in abstract)	IBA	Genetic fidelity confirmed; complete protocol	Yes (HPTLC)
Present study (2022)	Leaf	0.5 mg/L BAP + 0.4 mg/L NAA	Not achieved	0.8 mg/L NAA	78.3% callus induction; 65.7% rooting; phytochemical screening	No (limitation)

Table 4 summarizes key methodological details and outcomes from major A. indica* tissue culture studies. PGR = plant growth regulator; AA = aristolochic acid; HPTLC = high-performance thin-layer chromatography.*

Several important observations emerge from this comparison:

1. Consistency in Optimal PGR Combinations: There is remarkable consistency across studies regarding the effectiveness of BAP-NAA combinations for *A. indica* tissue culture. Most studies report optimal shoot regeneration with BAP concentrations of 0.8-2.0 mg L^{-1} combined with NAA at 0.4-0.5 mg L^{-1}, corresponding to BAP:NAA ratios of 1.6:1 to 4:1. This consistency validates the reproducibility of these protocols and suggests that *A. indica* has a relatively narrow optimal PGR range for shoot regeneration.

2. Explant Source Matters: Studies using nodal explants (Manjula et al., 1997; Dey et al., 2021) have generally achieved higher regeneration frequencies and more complete protocols compared to those using leaf explants. This is consistent with the general principle that nodal explants, which contain preexisting meristematic tissue, are more responsive to *in vitro* culture than leaf explants, which require dedifferentiation before organogenesis can occur (George et al., 2008)

3. Protocol Completeness Varies: Only a few studies (Manjula et al., 1997; Dey et al., 2021) have reported complete protocols including callus/shoot induction, rooting, and successful acclimatization with

quantitative survival data. The present study, like several others, represents a preliminary investigation that requires further optimization to achieve complete plantlet regeneration.

4. Aristolochic Acid Assessment is Rare: Critically, only one study (Dey et al., 2021) has included a quantitative assessment of aristolochic acid content in tissue culture-derived material. This represents a major gap in the literature, as AA content is the most important safety parameter for any Aristolochia propagation protocol. The present study shares this limitation and highlights the urgent need for future work to include AA quantification.

5. Genetic Fidelity Assessment is Emerging: Dey et al. (2021) represent an important advancement by incorporating molecular marker analysis (RAPD, ISSR) to confirm the genetic fidelity of micropropagated plants. This addresses a critical concern in conservation biotechnology, as tissue culture can induce somaclonal variation that may compromise the genetic integrity of conserved germplasm (Neelakandan & Wang, 2012). Future protocols should routinely include genetic fidelity assessment, particularly for conservation applications.

4.6 Broader Context: Conservation Biotechnology and Medicinal Plant Production

The development of *in vitro* propagation protocols for threatened medicinal plants, such as A. indica, must be situated within the broader context of conservation biotechnology and sustainable medicinal plant production (Rout et al., 2000; Chen et al., 2016).

Conservation Biotechnology: Plant tissue culture offers several advantages for ex situ conservation of threatened species, including: - Rapid multiplication of large numbers of plants from limited source material - Year-round production independent of seasonal constraints - Production of disease-free planting material - Long-term germplasm storage through cryopreservation - Potential for genetic improvement through somaclonal variation or genetic transformation

However, successful conservation biotechnology requires more than just protocol development. It must be integrated with in situ conservation efforts, including genetic diversity assessment to ensure representative sampling of populations, genetic fidelity testing to confirm clonal stability, and demonstration of successful reintroduction or population augmentation in natural habitats (Sarasan et al., 2006). The present study represents only the first step in this process and requires substantial additional work to achieve these broader conservation goals.

Sustainable Medicinal Plant Production: For species with legitimate medicinal value and acceptable safety profiles, *in vitro* propagation can help sustain production by reducing pressure on wild populations. However, for Aristolochia species, the presence of toxic aristolochic acids fundamentally changes this calculus. Rather than supporting increased production for medicinal use, biotechnological efforts should focus on: - Developing AA-reduced or AA-free cultivars through screening or metabolic engineering - Identifying and isolating specific beneficial compounds (flavonoids, terpenoids) for pharmaceutical development - Exploring alternative species or synthetic compounds that provide similar therapeutic benefits without toxicity risks

4.7 Limitations of the Present Study

Several limitations of the present study must be acknowledged:

1. Incomplete Protocol: The most significant limitation is the lack of shoot regeneration from callus, which prevents the production of complete plantlets via indirect organogenesis. Future work must systematically evaluate cytokinin-dominant PGR combinations to achieve shoot induction.

2. No Aristolochic Acid Quantification: The study did not include a quantitative assessment of AA content in plant material or tissue culture-derived callus. This represents a critical gap that must be addressed in future work using HPLC, LC-MS, or HPTLC methods.

3. No Genetic Fidelity Assessment: The study did not include molecular marker analysis to assess the genetic stability of callus cultures. Prolonged callus culture can induce somaclonal variation, which may be undesirable for conservation applications but could be exploited to select low-AA variants.

4. Qualitative Phytochemistry Only: The phytochemical screening was purely qualitative, providing presence/absence data but no information on specific compound identities or quantitative concentrations.

Future work should employ advanced analytical techniques (HPLC, GC-MS, LC-MS) for comprehensive metabolite profiling.

5. No Acclimatization Data: The study did not include the development of an acclimatization protocol or assessment of ex vitro survival rates. This is essential for demonstrating the practical utility of the propagation system.

6. Limited Replication: While the study included three independent experiments with 20 explants per treatment (n = 60), larger sample sizes would provide greater statistical power to detect subtle differences between treatments.

7. No Comparison with Field-Grown Plants: The study did not compare the phytochemical profiles or growth characteristics of tissue-culture-derived material with those of field-grown plants, which would have provided insights into the effects of *in vitro* culture conditions on plant metabolism and development.

4.8 Future Directions

Based on the findings and limitations of the present study, several important directions for future research can be identified:

1. Complete the Regeneration Protocol: - Systematically evaluate cytokinin-dominant PGR combinations for shoot induction from callus - Optimize shoot multiplication and elongation - Develop a robust acclimatization protocol with quantitative survival data - Assess growth and morphological characteristics of regenerated plants.

2. Quantify Aristolochic Acid Content: - Develop and validate HPLC or LC-MS methods for AA quantification in *A. indica* - Measure AA content in field-grown plants (roots, stems, leaves) - Measure AA content in tissue culture-derived callus and regenerated plants - Investigate factors influencing AA accumulation (plant age, tissue type, environmental conditions, culture conditions).

3. Screen for Low-AA Variants: - Generate large populations of micropropagated plants - Screen for natural or somaclonal variants with reduced AA content - Assess genetic stability of low-AA lines - Investigate the genetic and biochemical basis of AA variation.

4. Assess Genetic Fidelity: - Employ molecular markers (RAPD, ISSR, SSR, or SNP) to assess the genetic stability of tissue culture-derived plants - Conduct flow cytometry to detect ploidy changes - Evaluate morphological and phytochemical stability across multiple subculture cycles

5. Comprehensive Phytochemical Profiling: - Employ HPLC, GC-MS, and LC-MS for identification and quantification of specific compounds - Compare metabolite profiles of field-grown plants versus tissue culture-derived material - Investigate the effects of culture conditions (PGRs, light, nutrients, elicitors) on secondary metabolite production - Conduct bioactivity assays (antioxidant, antimicrobial, anti-inflammatory) to correlate chemical composition with pharmacological activities.

6. Explore Metabolic Engineering Approaches: - Identify genes involved in aristolochic acid biosynthesis - Develop genetic transformation or gene editing protocols for *A. indica* - Attempt to knock out or down-regulate AA biosynthesis genes while maintaining production of beneficial metabolites - Assess the feasibility of producing "safe" *A. indica* cultivars.

7. Integrate with Conservation Strategies: - Assess genetic diversity of wild *A. indica* populations using molecular markers - Develop sampling strategies to capture representative genetic diversity in *ex situ* collections - Conduct reintroduction or population augmentation trials - Monitor long-term survival and reproductive success of reintroduced plants.

5. CONCLUSION

This study successfully established a preliminary *in vitro* propagation protocol for *Aristolochia indica L.*, a medicinally important yet threatened plant species. Among the plant growth regulator combinations assessed, MS medium supplemented with 0.5 mg L^{-1} BAP and 0.4 mg L^{-1} NAA was identified as optimal for callus induction from leaf explants, achieving 78.3 ± 4.2% callus induction frequency and 1.24 ± 0.18 g fresh weight after 28 days of culture. Subsequent root organogenesis was successfully induced on MS medium containing 0.8 mg L^{-1} NAA, with 65.7 ± 5.1% rooting efficiency. Preliminary phytochemical

screening confirmed the presence of diverse secondary metabolite classes, including flavonoids, tannins, terpenoids, alkaloids, phenolics, saponins, and proteins/amino acids, reinforcing the species' phytochemical diversity

However, several critical limitations must be acknowledged. First, the protocol is incomplete, as shoot regeneration from callus was not achieved in the present study. Future work must focus on optimizing shoot induction using cytokinin-dominant PGR combinations, developing a complete acclimatization protocol, and demonstrating successful *ex vitro* establishment. Second, and most importantly, the study did not include a quantitative assessment of aristolochic acid content, which is the most critical safety parameter for any work involving *Aristolochia* species.

The presence of aristolochic acids in *A. indica* has been confirmed by multiple previous studies, and these compounds are known to cause severe nephrotoxicity and urothelial carcinoma in humans. Regulatory agencies worldwide have banned or restricted the use of *Aristolochia*-containing products due to the serious health risks they pose. Therefore, any future application of this propagation protocol must include a comprehensive quantification of aristolochic acid and risk assessment. The traditional medicinal uses of *A. indica* cannot be promoted without addressing these safety concerns, and conservation efforts should be clearly separated from medicinal use claims.

Future research directions should include: (1) completion of the regeneration protocol with shoot induction and acclimatization, (2) quantitative assessment of aristolochic acid content in tissue culture-derived material using HPLC or LC-MS, (3) genetic fidelity assessment using molecular markers, (4) comprehensive phytochemical profiling using advanced analytical techniques, and (5) exploration of biotechnological approaches to develop aristolochic acid-reduced or AA-free cultivars. Only through such comprehensive investigations can the potential of *in vitro* propagation for the conservation and safe utilization of *A. indica* be fully realized.

This study contributes to the foundational knowledge base for *A. indica* tissue culture while highlighting the critical importance of addressing safety concerns in all work involving *Aristolochia* species. The protocol developed here provides a starting point for future optimization efforts, but substantial additional work is required before it can be considered suitable for conservation or commercial applications.

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