SPECIMEN FORMAT FOR THESES OF MONTH

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Department	**:**	Botany
Branch/ Area:	**:**	Plant Biotechnology and Phytochemistry
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Title of the thesis	**:**	Enhancing *In Vitro* Propagation Efficiency and Exploring Wound Healing Therapeutic Potential of *Rauvolfia tetraphylla* L.: A Multifaceted Approach
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Designation of Supervisor	**:**	Assistant Professor
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Abstract within 300 words:

Rauvolfia tetraphylla L., a medicinal plant from the Apocynaceae family, is renowned for its traditional pharmacological uses, especially in treating skin ailments and wounds. However, habitat destruction and overharvesting have posed serious threats to its survival. Due to its low seed germination rate, this study focused on conserving *R. tetraphylla* through improved germination and micropropagation techniques. Pretreating seeds at 4°C for 48 hours and precutting the seed coat achieved an 83.33% germination rate. Callus formation was successfully induced from leaf, node, internode, and root explants using different hormone combinations. Regeneration occurred via both direct organogenesis and somatic embryogenesis, providing a foundation for tissue culture protocols and conservation.

The wound-healing properties of ethyl acetate and methanol extracts from *R. tetraphylla* leaves and fruits were systematically evaluated through *in vitro* scratch assays and *in vivo* zebrafish caudal fin regeneration. Extracts at 100 µg/mL promoted rapid cell migration and enhanced fin regeneration within 21 days. Histological analysis confirmed higher cellular regeneration in tireated groups compared to controls.

Alkaloids were linked to enhanced wound healing, prompting the identification and characterization of the tryptophan decarboxylase (*RtTDC*) gene involved in alkaloid biosynthesis. The 1,500 bp *RtTDC* gene encodes a 499-amino-acid protein closely related (95.3%) to *R. verticillata*. Its enzymatic activity was confirmed through molecular docking with L-tryptophan and HPLC analysis.

Bioactive compounds identified via GC-MS and LC-MS were further analyzed through molecular docking using protein structures (PDB IDs: 6Y8M, 6B8Y, 1GEN), revealing strong interactions and potential wound-healing activity. These findings highlighted *R. tetraphylla* therapeutic potential and advocate for further research, including clinical trials, to validate its efficacy as a wound-healing agent.

i) Major objectives :

To overcome the lacuna in enhancing seed propagation, an efficient strategy is strongly required. Hence, we have been proposed a method for propagation as well as conservation

and understanding the molecular basis of alkaloid biosynthesis. Based on the ethnopharmacological study *Rauvolfia tetraphylla* is used to treat wound in folk medicine. Despite the recognized potential, there remains a dearth of studies exploring the wound healing capabilities of this plant extract. Consequently, this research has the potential to address the therapeutic gap crucial for effective wound healing.

- To optimize the seed germination potential for *Rauvolfia tetraphylla*, and to investigate the effect of PGR on callus induction, root and shoot formation from different explants, subsequently examining the histological aspects of somatic embryogenesis derived from the callus.
- To identify and characterize the major bioactive compounds in *Rauvolfia tetraphylla* using spectroscopic chromatographic techniques.
- To assess the biological activity of various crude extracts from leaves and fruits from *Rauvolfia tetraphylla* for assessing wound healing properties.
- To isolate and characterize the Tryptophan Decarboxylase (TDC) gene from *Rauvolfia tetraphylla* (RtTDC), investigating its sequence, structure, catalytic regions, evolutionary relationships, physicochemical properties, and substrate recognition, followed by experimental validation of its catalytic activity through a TDC assay.
- To perform docking analysis of the bioactive compounds of *Rauvolfia tetraphylla* against a wound healing protein (6Y8M, 6B8Y and 1GEN).

ii) Hypothesis:

Rauvolfia tetraphylla possesses significant wound-healing potential due to its bioactive alkaloid content; however, limited seed germination and propagation hinder its widespread utilization. Therefore, the development of an efficient micropropagation strategy alongside molecular characterization of alkaloid biosynthesis pathways, particularly involving the TDC gene which will facilitate both conservation of the species and validation of its therapeutic potential, especially in wound healing as supported by ethnopharmacological evidence.

iii) Methodology :

The present investigation, "Enhancing *In Vitro* Propagation Efficiency and Exploring Wound Healing Therapeutic Potential of *Rauvolfia tetraphylla* L.: A Multifaceted Approach", was carried out.

Plant Tissue Culture

Glassware was cleaned using chromic acid, washed, and sterilized. Saplings of *R. tetraphylla* were collected from Top Slip, Pollachi, and authenticated by BSI (BSI/SRC/5/23/2023/Tech-552). Murashige and Skoog (1962) medium was used as the basal culture medium, supplemented with 3% sucrose, 0.1% meso-inositol, 0.8% agar, and pH adjusted to 5.7 before autoclaving. Auxins (2,4-D, IAA, IBA) and cytokinins (BAP, KIN) were freshly prepared and stored at 4°C.

Sterilized seeds were pre-treated at 4°C for 2 days and inoculated onto hormone-free MS media. Explants (leaf, stem, node, internode, root) from in vitro-raised seedlings were cultured on MS medium with various PGR combinations. Culture conditions included 25±2°C, 16/8 h photoperiod, and 2000 lux light intensity. Callus induction was monitored from day 7 and subcultured every 21 days. Rooting (direct rhizogenesis) and shoot formation (caulogenesis) were assessed using IAA, IBA, and BAP. Histological analysis of somatic embryogenesis was performed by fixing samples in FAA, embedding in paraffin, sectioning, staining with safranin, and observation under a light microscope. Rooted plantlets were acclimatized using sterilized soil and vermiculite (1:2 ratio). Statistical analysis was analysed by one-way ANOVA, and significance was determined by Duncan's Multiple Range Test at $p \leq 0.05$.

Phytochemical Analysis

Shade-dried leaves and fruits were powdered and macerated with solvents (hexane, chloroform, ethyl acetate, methanol). Extracts were concentrated using a rotary evaporator and stored for further studies. Qualitative
phytochemical screening included tests for alkaloids, carbohydrates, glycosides, saponins, steroids, terpenoids, phenolics, flavonoids, tannins, fixed oils, and proteins using standard colorimetric and precipitation-based methods.

Quantitative Phytochemical Estimations

Methanol and ethyl acetate extracts were used for quantifying tannins, alkaloids, flavonoids, and phenolics.

- Tannins were determined using the Folin–Ciocalteu method and expressed as µg gallic acid equivalent (GAE)/mg.

- Alkaloids were quantified with bromocresol green at 470 nm and calculated as µg atropine equivalent/mg.
- Flavonoids were analyzed using aluminium chloride colorimetry and expressed as µg rutin equivalent/mg.
- Phenolics were measured via Slinkard & Singleton's method and calculated in µg GAE/mg.

Chromatographic and Spectral Analysis

Methanolic leaf extracts (2 g) were fractionated by column chromatography using increasing polarity solvents (hexane to methanol). Eluted fractions were evaluated by TLC on silica gel plates and visualized under UV light (254/365 nm) for R_f value determination.

The most bioactive fraction was subjected to spectral characterization using:

- UV-Vis spectrophotometer (Labman)
- FTIR (Shimadzu)
- GC-MS/MS (Perkin Elmer Clarus 680)
- NMR (Bruker 500 MHz) for ^{1}H and ^{13}C

Biological activities

The study investigates the wound-healing potential of *R. tetraphylla* through Multifaceted approaches.

- Antibacterial activity was tested using agar well diffusion against *E. coli, S. aureus,* and *E. faecalis,* with ethyl acetate and methanol extracts of leaves and fruits. MICs were determined via broth and agar dilution.
- Antioxidant activity was assessed using DPPH, phosphomolybdenum, and FRAP assays, with IC_{50} values calculated.
- Anti-inflammatory potential was evaluated using egg albumin denaturation assay. Extracts (20–120 µg/mL) were compared against a diclofenac control to assess protein stabilization.
- Cytotoxicity was analyzed in Vero and 3T3 cell lines using MTT assay; viability and CC_{50} were determined across a concentration range (5–100 µg/mL).

Chick CAM assay assessed angiogenesis inhibition by placing extract-soaked filter paper discs on the CAM of 8-day-old chick embryos and observing blood vessel branching by day 12.

- Heat stress resistance was evaluated in *Caenorhabditis elegans*, where survival was monitored after exposure to 35°C heat shock for 180 minutes on extract-treated NGM plates.
- Wound healing potential was explored using a scratch assay, where cell migration and wound closure were measured microscopically over 24 h.
- *In vivo* wound healing studies utilized zebrafish (TL/Ek strain). Caudal fins were injured and treated with extracts (25–100 µg/mL). Regeneration was monitored over 21 days, followed by histological examination of re-epithelialization and tissue development.

Characterization of TDC gene

Molecular studies included isolation and PCR amplification of the *RtTDC* gene using gene-specific primers, followed by cloning into pTZ57R/T vector, transformation into *E. coli*, and confirmation via colony PCR and sequencing. The gene product's structure was analyzed using SWISS-MODEL, BLAST, and MEGA for phylogeny. Physicochemical and structural properties were predicted using ProtParam, HNN, and hydropathy plots. Ligand-receptor interaction studies involved docking L-tryptophan with the *RtTDC* protein and analyzing interactions using AutoDock Vina, PyMOL, and LigPlot+.

Molecular docking

Docking analyses were determined on IL-1β, TGF-β, and MMP9 receptors with bioactive ligands identified from GC-MS/LC-MS profiles.

iv) Findings:

[1] This comprehensive study on *Rauvolfia tetraphylla* has yielded valuable insights across diverse facets, including tissue culture optimization, phytochemical composition analysis, wound-healing potential assessment and molecular characterization of the Tryptophan Decarboxylase (TDC) gene.

[2] The refined tissue culture protocols, with a focus on somatic embryogenesis, contribute to efficient micropropagation and regeneration strategies.

[3] The phytochemical analysis identified bioactive compounds, suggesting medicinal potential, while the wound-healing investigations demonstrated antimicrobial efficacy, antioxidant activity and regenerative effects both *in vitro* and *in vivo*.

[4] The molecular characterization of the TDC gene provides crucial information on alkaloid biosynthesis and molecular docking studies shed light on po tential interactions of *R. tetraphylla* compounds with proteins associated with wound healing.

[5] Collectively, these findings advance our understanding of *R. tetraphylla* biology and also offer promising avenues for applications in plant tissue culture, phytochemical analysis, biological activity and the development of therapeutic agents for wound healing.

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