

CHAPTER V

SUMMARY AND CONCLUSION

This chapter provides a summary of the study along with conclusion. Two research gaps were analysed in this study. The first pertains to the plant's endangered status attributed to its poor germination rate. To overcome this issue an efficient, standardized, and optimized protocol has been developed using plant tissue culture techniques. The second gap concerns the ethnobotanical use of *R. tetraphylla* for wound healing without scientific evidence. To address this discrepancy, our study focused on analysing the wound healing properties of different crude extracts of *R. tetraphylla*.

The effect of surface sterilization on *R. tetraphylla* seeds using sodium hypochlorite (NaHCl₃) and mercuric chloride (HgCl₂) solutions at varying concentrations and durations were investigated. The results indicated that increasing the duration of treatment with NaHCl₃ and HgCl₂ led to higher percentages of sterile plantlets. Specifically, treatments with longer exposure times resulted in greater percentages (98.44%) of sterile plantlets, with the maximum germination percentage achieved using 20 minutes of NaHCl₃ and 3 minutes of HgCl₂. The surface sterilization process effectively reduced microbial contamination, thereby improving the likelihood of successful germination and growth of *R. tetraphylla* seeds. It emphasizes the importance of meticulous sterilization protocols in ensuring the viability and health of plant materials for further experimentation.

Improved the germination rate of *R. tetraphylla* seeds by addressing the hindrance posed by their naturally occurring hard seed coat. Through a combination of surface sterilization, cold pre-treatment, and partial removal of the seed coat, this study achieved significant enhancement in germination rates under *in vitro* conditions. The cold treatment effectively broke seed dormancy, while cutting one edge of the seeds facilitated germination by circumventing the barrier imposed by the hard seed coat. The results showed that 83.33% of the cold pre-treated, pre-cut seeds were germinated on agar-solidified MS medium with 3% sucrose, while uncut seeds remained dormant. It emphasizes the importance of addressing seed coat impermeability to enhance

germination rates in *R. tetraphylla* seeds under controlled conditions. By overcoming the barrier imposed by the hard seed coat, the technique facilitates seed germination and seedling development, providing a valuable method for propagating *R. tetraphylla* for further research.

To evaluate the impact of various plant growth hormones (IAA, IBA, 2,4-D, and BAP) and their combinations on callus formation in leaves, node, internode, and root explants of *R. tetraphylla*. Gradual increases in callus formation were observed with increasing concentrations of individual hormones. Combinations of hormones further enhanced callus induction compared to individual growth regulators. The maximum callus formation in leaf (88.62%), node (83.45%), internode (79.66%) and root (84.11%) explants were achieved on MS medium with a combination of 2,4-D + BAP (2.5 mg/L each). These results demonstrated the significant influence of specific hormone combinations and concentrations on callus formation of *R. tetraphylla*.

To analyses direct organogenesis of root induction from leaves and nodal explants of *R. tetraphylla* on MS medium with different concentration of PGRs (IAA, 2,4-D, IBA, and BAP). Increasing concentrations of IAA led to higher percentages of direct root formation, with a similar trend observed for hormone combinations like 2,4-D+BAP, IAA+BAP, and IBA+BAP. The maximum percentages of direct root formation from leaf (87.50%) and node (83.16%) were achieved on MS medium with the combinations of IAA (1.5 mg/L) + BAP (0.5 mg/L). It emphasizes effect on PGRs and their concentrations on direct root formation from leaf and nodal explants of *R. tetraphylla*.

The effect of PGRs on direct shooting and multiple shoot formation in nodal explants of *R. tetraphylla* were investigated on MS medium with BAP, 2,4-D+BAP, IAA+BAP, and KIN+BAP. Results indicated that increasing concentrations of BAP had a significant impact on both direct shooting and multiple shoot formation. Similarly, combinations of 2,4-D+BAP, IAA+BAP, and KIN+BAP also demonstrated positive effects on both shooting parameters. The maximum percentage of direct shoot (83.44%) and multiple shoot (88.44%) was achieved on MS medium with IAA + BAP (1.5 mg/L each). It indicates the importance of hormone concentration optimization in promoting direct and multiple shoot formation of *R. tetraphylla* nodal explants.

The impact of various PGRs on somatic embryogenesis in leaf and root explants were evaluated and the preliminary experiments identified 2,4-D and BAP are the efficient auxin and cytokinin at an optimal concentration of 2.5 mg/L. Further experiments focused on leaf and root explants inoculated on MS medium with different concentrations of 2,4-D + BAP. After a 4-week culture period, the maximum embryogenesis was observed with a combination of BAP + 2,4-D (1.5 mg/L), indicating a synergistic effect conducive for somatic embryo development. It established the potential for achieving plant regeneration through somatic embryogenesis, offering insights into optimizing tissue culture protocols for efficient somatic embryo production.

Histological analysis elucidated the cellular dynamics during somatic embryogenesis in *R. tetraphylla*, shedding light on the progression of embryogenic and non-embryogenic cell populations. The identification of distinct cell types and developmental stages provides a foundation for understanding the mechanisms underlying *in vitro* plant regeneration.

The qualitative preliminary phytochemical analyses of *R. tetraphylla* leaf and fruit extracts using different solvents revealed the presence of various phytoconstituents. These include alkaloids, carbohydrates, glycosides, oils and fats, phenolic compounds, tannins, quinones, cardiac glycosides, terpenoids, coumarins, phlobatannins, steroids, phytosteroids, anthraquinones, flavonoids, and proteins across all the extracts. The detection of various secondary metabolites in *R. tetraphylla* leaf and fruit extracts suggests their potential pharmacological and therapeutic significance. These compounds may contribute to the medicinal properties associated with *R. tetraphylla*, including its traditional uses in herbal medicine.

The alkaloid, flavonoid, total phenolic content, and tannin, of *R. tetraphylla* leaf and fruit extracts using ethyl acetate and methanol crude extracts were determined by different chemical assays. Gallic acid (tannins and phenols), atropine (alkaloids), and rutin (flavonoids) were used as standard. The maximum alkaloids (2.10 µg/mg), flavonoids (1.92 µg/mg), and tannin content (33.92 µg/mg) was observed in methanolic extract of leaf, and the maximum total phenolic content (22.88 µg/mg) was identified in the methanolic extract of fruit. The presence of bioactive compounds such as alkaloids, flavonoids, phenols, and tannins suggested that *R. tetraphylla* possess antioxidant,

antimicrobial, and other health-promoting properties. Additionally, the variation in phytochemical content among different plant parts and extraction solvents underscores the importance of standardized extraction protocols for obtaining consistent and reliable results in phytochemical analysis.

GC-MS analysis of the methanolic extract of *R. tetraphylla* showed the presence of twelve compounds, each with specific percentages of abundance. These compounds include 1,9-Nonanediol, 3-Furanol, tetrahydro-2,2,4,4-tetramethyl-, 1D-1-O-Methyl-myo-inositol, (Z)-2-methylbutanal oxime, Arabino-heptitol, “2,3:5,6-dianhydro-1,7-dideoxy-2,6-di-C-methyl-, 3-O-methyl-D-glucose, Butyl cyclohexyl phthalate, 7-[3-Chloro-2-hydroxypropyl] guanine, 1-Naphthalenepropanol, alp, 1,3,3-trimethyl-2-hydroxymethyl-3,3-dimethyl-4-(3-methylbut-2-enyl)-cyclohexene, β -Amyrone, and (+)-Helminthogermacrene.

The presence of diverse compounds in the methanolic extract of *R. tetraphylla* suggested that potential therapeutic applications. Compounds such as 1D-1-O-Methyl-myo-inositol, known for addressing metabolic disorders like insulin resistance and diabetes-related complications, and 3-O-methyl-D-glucose, with potential applications in tumour detection and stroke management, indicate the pharmacological significance of *R. tetraphylla*. Additionally, compounds like Butyl cyclohexyl phthalate, 7-[3-Chloro-2-hydroxypropyl] guanine, β -Amyrone, and (+)-Helminthogermacrene exhibit antioxidant, anticancer, anti-inflammatory, and antiviral activities, suggesting the potential of *R. tetraphylla* in treating various ailments. The identified compounds contribute to the antioxidant, antimicrobial, anti-inflammatory, and wound healing activities attributed to *R. tetraphylla*. Further research will be focusing on isolation and characterization of these compounds and enhance efficacy for drug development.

The LCMS analysis of the methanol extract of *R. tetraphylla* leaves identified nine significant peaks corresponding to secondary metabolites. These compounds include Oxodecenoic acid, Naringenin, dihydrokaempferide, serpentine, yohimbine, isoreserpine, darcyrine, reserpiline, carapanubine, and reserpine, each with distinct pharmacological properties and potential therapeutic applications. Compounds like dihydrokaempferide, serpentine, yohimbine, and reserpine exhibited a range of pharmacological activities including antioxidant, anti-inflammatory, antipsychotic, and

antihypertensive properties, indicating the potential of *R. tetraphylla* in treating various ailments such as acute pancreatitis, liver inflammation, erectile disorder, and hypertension. The wound healing properties attributed to these secondary metabolites emphasize the traditional medicinal uses of *R. tetraphylla* and its potential applications in modern healthcare practices.

The antimicrobial activity of *R. tetraphylla* ethyl acetate and methanolic extracts against Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative (*E. coli*) bacteria were evaluated using the agar well diffusion method. Both extracts exhibited significant antimicrobial activity, with the methanol extract showing the highest inhibition (19 mm) against *E. coli*. The MIC against *E. coli* was determined using a two-fold broth dilution method, with concentrations ranging from 0.78 µg/mL to 200 µg/mL. The ethyl acetate and methanol extracts of *R. tetraphylla* leaves and fruits demonstrated varying MIC values against *E. coli*. It confirmed the antimicrobial potential of *R. tetraphylla* extracts, supporting its traditional medicinal uses in treating microbial infections.

The *in-vitro* antioxidant activity of ethyl acetate and methanol extracts from *R. tetraphylla* leaf and fruit was evaluated using various assays such as DPPH, FRAP, and total antioxidant activity. DPPH scavenging activity of *R. tetraphylla* showed maximum antioxidant activity in methanol extract of fruit (IC₅₀ = 60.37 µg/mL) followed by methanol extract of leaf (IC₅₀ = 62.61µg/mL). In the FRAP assay, the extracts demonstrated the ability to reduce Fe³⁺ to Fe²⁺ ions, with a clear dose-response relationship observed across different concentrations. The methanolic extract from the leaves exhibited the highest antioxidant activity among all extracts tested. The sequence of antioxidant potential observed was RLM < RL EA < RFM < RF EA. Furthermore, the total antioxidant activity revealed that the methanol extract of the leaf showed maximum (IC₅₀ = 63.05 µg/mL) antioxidant activity, followed by methanol extract of the fruit (IC₅₀ = 65.37 µg/mL). It emphasizes the significant antioxidant potential of *R. tetraphylla* leaf and fruit extracts, as demonstrated by their potential for wound healing activity.

The anti-inflammatory activity of *R. tetraphylla* leaf and fruit extracts was estimated by the denaturation of egg albumin method, an *in vitro* technique assessed anti-inflammatory properties by inhibiting albumin protein denaturation. The methanol

extracts from both the leaf ($IC_{50} = 72.44 \mu\text{g/mL}$) and fruit ($IC_{50} = 78.04 \mu\text{g/mL}$) exhibited the maximum inhibition rate at a concentration of $100 \mu\text{g/mL}$, with inhibition increasing gradually with concentrations. *R. tetraphylla* leaf and fruit extracts possess anti-inflammatory properties, it is warranted to elucidate the underlying mechanisms of action and to evaluate the safety and efficacy of these extracts for wound healing property.

The cytotoxicity and cell viability of ethyl acetate and methanol extracts from *R. tetraphylla* leaves and fruits against Vero cells and 3T3 mouse embryonic fibroblast cells. The MTT assay was employed to assess quantitative cell viability, revealed the concentration-dependent cytotoxic effects of *R. tetraphylla* extracts. Results indicated that all extracts exhibited CC_{50} values greater than $100 \mu\text{g/mL}$, suggested that minimal cytotoxicity and better proliferation activity towards both cell lines within the tested concentration range. Henceforth, *R. tetraphylla* extracts possess relatively low cytotoxicity against Vero and 3T3 cell lines and their potential applications in therapeutic wound healing properties inducing without any significant toxicity.

Cell migration is a critical aspect of wound healing, was evaluated using Vero and 3T3 cell lines treated with ethyl acetate and methanol crude extracts of *R. tetraphylla*. Wounds were created in Vero and 3T3 monolayers treated with test samples, and observed after a 24-hour incubation period, showed a decrease in wound distance indicating wound closure and cell migration. Wound closures were evaluated in the sample group with the highest percentage (72.67%) was observed in methanol extract of leaves. It exemplified the wound-healing properties of *R. tetraphylla* extracts, as increased cell migration and wound closure rates compared to the control group. It premises their potential therapeutic applications in promoting tissue repair and wound healing.

The CAM assay was determined using the *in-ovo* method to assess the angiogenic potential of ethyl acetate and methanol extracts derived from the leaf and fruit of *R. tetraphylla*. Results indicated that ethyl acetate extracts exhibited limited angiogenic effects, methanol extracts demonstrated significant angiogenic property. Qualitative assessment of vascular morphology revealed blood vessels present on materials and showed complete enclosure of the collagen/elastin membrane by the chorioallantoic

membrane. These extracts exhibited notable effects on angiogenesis, a critical process in wound healing.

Ethyl acetate and methanol extracts from leaf and fruit of *R. tetraphylla* were assessed for their ability to resistance against heat stress in *Ceanorhabditis elegans*. Results demonstrated that higher survival rates among the worms, indicating a protective effect against heat stress. Among the extracts tested, methanol from the leaf exhibited the highest survival rate (22 days), followed by fruit (21 days) extracts. It suggested the adaptogenic properties of *R. tetraphylla* extracts and their potential application in mitigating the effects of heat stress-related conditions.

The wound healing potential of ethyl acetate and methanol extracts derived from the leaf and fruit of *R tetraphylla* was analysed by a Zebra fish model. Fish were grouped into control and treated, with the treated groups receiving various concentrations of the extracts. Incisional wounds were created on the caudal fins of the fish, and wound healing progression was monitored over a period of 21 days. Results showed that fish treated with 100 µg/mL of ethyl acetate and methanol extracts exhibited maximum fin regeneration by the 21st day compared to the control group. Histological analysis through haematoxylin and eosin staining confirmed enhanced wound healing in the treated groups, with increased neutrophil counts observed compared to the control group. It also revealed various cellular changes associated with wound healing including re-epithelialization, fibroblast activity, and tissue remodelling.

Tryptophan decarboxylase gene from *R. tetraphylla* (*RtTDC*) was investigated for its potential role in the biosynthesis of monoterpene indole alkaloids (MIAs) and its implications for wound healing. The TDC gene sequence was obtained from *R. tetraphylla* and comparative genomic analysis revealed a similarity index of 95.39% with the TDC gene from *R. verticillata*, suggested the conservation within the *Rauvolfia* genus. Structural modelling of *RtTDC* was examined by crystallographically structure of *C. roseus* TDC. The homology model exhibited high-quality features, indicating structural similarities with known TDC proteins. Moreover, phylogenetic analysis highlighted the evolutionary relationships between TDC sequences from different species.

Physicochemical properties of *RtTDC* were predicted, revealing insights into its biophysical characteristics, stability, and solubility. Leucine is the most abundant amino acid observed in TDC sequence. Secondary structure analysis indicated a prevalence of alpha helices, further characterizing the conformational features of *RtTDC*. Molecular docking studies with L-tryptophan as the substrate suggested potential interactions and binding affinity between *RtTDC* and its substrate.

Enzymatic decarboxylation activity assays confirmed *RtTDC* role in catalytic activity, which convert L-tryptophan to tryptamine, a crucial step in alkaloid pathways. Identification and characterization of *RtTDC* provide valuable insights into the molecular mechanisms underlying alkaloid biosynthesis in *R. tetraphylla*. The investigation of the TDC gene from *R. tetraphylla* sheds light on its potential involvement in the biosynthesis of indole alkaloids and its significance in wound healing processes.

Bioactive compounds identified through GCMS and LCMS analysis were subjected to molecular docking studies suggested that potential pharmacological implications of *R. tetraphylla* in drug design and development. *In silico* docking studies were accomplished using three different protein structures (PDB IDs: 6Y8M, 6B8Y, 1GEN) with various ligands selected for their potential in wound healing applications. The binding affinities of the ligands were evaluated, and their interactions with amino acid residues within the protein structures were analysed.

Protein structure with PDB ID 6Y8M, compounds such as Butyl cyclohexyl phthalate (-7.67 kcal/mol), and Isoreserpine (-8.25 kcal/mol) showed stronger binding affinities. Similarly, in the protein structure with PDB ID 6B8Y, Butyl cyclohexyl phthalate (-7.70) and Carapanaubine (-8.63 kcal/mol) exhibited strong binding affinities. In this background of the protein structure with PDB ID 1GEN, 1-Naphthalenepropanol (-7.69), Carapanaubine (-8.22 kcal/mol) displayed strong binding affinities, while others like Butyl cyclohexyl phthalate (-6.29 kcal/mol), Serpentine (-6.83 kcal/mol), Yohimbine (-6.23 kcal/mol), and Isoreserpine (-5.98 kcal/mol) also formed significant interactions.

The docking studies provide insight the potentials of ligand-protein interactions within the given protein structures, contributing to a respective understanding of their pharmacological significance and potential therapeutic applications in wound healing. It contributes the molecular mechanisms underlying alkaloid metabolism in *R. tetraphylla*

and their implications for the development of novel therapeutic agents for wound healing property. Though, further research is required to investigate the clinical potential and safety profile of *R. tetraphylla* extracts to promote the formulation of wound healing drug.