
CHAPTER II

RIVIEW OF LITERATURE

This chapter focuses on consolidating past research and constructing a proposal for current work by reviewing literature on *Rauwolfia tetraphylla* and its related species. It encompasses a comprehensive overview of research conducted on *R. tetraphylla* from 1990 to 2024 which comprises medicinal uses, seedling development, plant tissue culture, extraction techniques, phytochemical screening, compound isolation, biosynthesis of alkaloids, biotechnological approaches as well as biological and pharmacological investigations such as antimicrobial, antioxidant, and anti-inflammatory properties, were evaluated across different crude extracts of *R. tetraphylla*.

2.1. Brief Description About the Plant

Indiscriminate trade of plant resources, uncontrolled collecting methods, habitat change, overexploitation, and climate change pose great threats to the availability of medicinal plants in most world countries, creating an urgent need for improved conservative methods and sustainable use of priority plant resources. As a result, *R. tetraphylla* has become an endangered species.

Plant alkaloids are chemically classified as amines and are defined by their function as bases generally containing one or more nitrogen atoms within a heterocyclic structure (Vezikov *et al.* 2024). Monoterpene Indole Alkaloids (MIAs) deserve the curiosity and attention of researchers due to their chemical diversity and biological activities and was reported in six Apocynaceae genera (Mohammed *et al.* 2021). These genera (*Alstonia*, *Rauwolfia*, *Kopsia*, *Ervatamia*, and *Tabernaemontana*, and *Rhazya*) consist of 400 members and represent 20% of Apocynaceae species (Mohammed *et al.* 2021). They further reported bioactivities, including antibacterial, antifungal, antioxidant, anti-inflammatory and immunosuppressive activities (Prasad *et al.* 2013).

Abdul-Hameed *et al.* (2022) investigated chromatographic profile from aerial parts of *Rhazya stricta* (Apocynaceae) and identified two new MIAs, 6-*nor*-antirhine-*N*₁-methyl razyamide. Alkaloids play a key role in biotic stress, abiotic stress, and physiological processes and protect plants against herbivores. The MIAs are one of the most structurally diverse families of alkaloids, with more than 2500 distinct molecules identified so far and are widespread within the Gentianales order including Apocynaceae, Gelsemiaceae, Loganiaceae, and Rubiaceae families (Stander *et al.* 2023).

Rauvolfia tetraphylla is a medicinal plant from the family Apocynaceae, native to India and other parts of Southeast Asia. It is widely used in traditional medicine to treat various ailments such as hypertension, mental disorders, fever, and malaria, blood pressure, wound, piles, diabetes, helminthiasis, insomnia, cough, skin diseases. The major bioactive compounds in *R. tetraphylla* are indole alkaloids such as reserpine, reserpiline, yohimbine, ajmaline, lankanescine, rauvotetraphyllines, alstonine, deserpidine, aricine, isoreserpine, sarpagine. These compounds are known for their pharmacological properties, including hypotensive, sedative, antipsychotic, and *in vitro* anti-cancer activities (Sabu *et al.* 2023; Balavaishnavi *et al.* 2024).

Plant-based drugs have been very popular and effective since ancient times, but thorough documentation with a scientific background on their effectiveness, the active chemical compounds present, and their action mechanisms is still scant (Luikham & Bhattacharyya, 2024). Tung *et al.* (2024) reported that alkaloids and lignans from the aerial parts of *R. tetraphylla* inhibit NO Production in LPS-activated RAW 264.7 Cells. Alzheimer's disease (AD) is a neurodegenerative disorder with no conclusive remedy. Yohimbine, found in *Rauvolfia vomitoria*, may reduce brain inflammation by targeting tumour necrosis factor-alpha (TNF α), implicated in AD pathogenesis (Tettevi *et al.* 2024). Stander *et al.* (2023) used metabolomics, proteomics, transcriptomics, and genome sequencing of *R. tetraphylla* with machine learning to discover the unexpected multiple actors of this natural product synthesis.

The antiarrhythmic drug ajmaline is a MIA isolated from the Ayurvedic plant *Rauvolfia serpentina* (Indian Snakeroot) (Guo *et al.* 2024). Lezin *et al.* (2024) reported a chromosome-scale genome assembly of *R. tetraphylla* facilitates identification of the complete ajmaline biosynthetic pathway. Wu *et al.* (2024) reported that Ulcerative colitis (UC) is an inflammatory disease in the digestive tract. *Rauvolfia* polysaccharide (Rau) has therapeutic effects on colitis in mice, and can inhibit the progress through the NOS2-mediated JAK2/STAT3 pathway (Wu *et al.* 2024). Reserpine, an alkaloid used for managing hypertension, has recently been identified as a potential drug for ameliorating retinal disorders (Lorrai *et al.* 2024).

Rauvolfia tetraphylla are represented by many synonyms, and distributed worldwide, especially in the Asian, and African continents. A total of seventeen *Rauvolfia* species have been traditionally explored for various therapeutic applications, out of which the roots of *R. serpentina* and *R. vomitoria* are used for the treatment of many diseases (Kumar *et al.* 2022). Due to its medicinal properties, the plant is widely cultivated in many parts of the world and

has gained significant attention from the scientific community for further research and development. It has various pharmacological properties that make it a promising candidate for research and development of new drugs.

Kumar *et al.* (2016) studied bioactive MIAs in ethanolic extract of seven *Rauvolfia* Species. The plant contains several important alkaloids, including reserpine, ajmaline, and serpentine, which have been extensively studied for their pharmacological properties. The most important alkaloids are reserpine which is used as an antihypertensive drug. According to Teleg *et al.* (2024) Reserpine (RES), a Vesicular Monoamine Transporter 2 (VMAT₂) inhibitor agent, has been used in preclinical research for many years to create animal models for depression and to test experimental antidepressant strategies.

Reserpine is a natural indole alkaloid, isolated from *R. serpentina*, and has potent antioxidant, antimicrobial, and anti-mutagenic properties (Ramu *et al.* 2021). About 287 alkaloids, seven terpenoids, nine flavonoids, and four phenolic acids have been reported in different parts of the forty-three species (Kumar *et al.* 2022). In addition to its medicinal properties, *R. tetraphylla* is also used for a variety of other purposes. The wood is hard and durable and is used for making tool handles and other small implements. The plant is also used as a hedge with significant medicinal and cultural importance in many parts of the world (Kumar *et al.* 2022).

2.2. Taxonomic Position

Kingdom: Plantae

Division: Angiospermae

Class: Dicotyledonae

Subclass: Gamopetalae

Series: Bicarpellatae

Order: Gentianales

Family: Apocynaceae

Genus: *Rauvolfia*

Species: *tetraphylla*

Binomial Name: *Rauvolfia tetraphylla* L.

(Synonyms: *R. canescens*; *R. heterophylla*; *R. hirsute*)

2.3. Vernacular Name

English : Wild snackroot, Devil pepper, Be still tree, American serpent wood

Hindi : Barachadrika, Chandrabhaga

Tamil	: Pampukaalaachchedi
Malayalam	: Pampumkolli, Kattamalpori
Telugu	: Papataku
Kannada	: Doddachandrike
Bengali	: Bar chandrika, gandhanakuli
Oriya	: Patalagarudi
Sanskrit	: Vanasarpagandha, Sarpanasini

2.4. Habit of *Rauvolfia tetraphylla*

Rauvolfia tetraphylla is native to the Indian subcontinent and is found in various regions of India, including the Western Ghats, Eastern Ghats, and the Himalayan foothills. It grows in a wide range of habitats such as evergreen forests, deciduous forests, grasslands, and along riverbanks, up to an altitude of about 1500 meters above sea level. It is also found in parts of Bangladesh, Sri Lanka, and some Southeast Asian countries (Mahalakshmi *et al.* 2019).



2.5. Botanical Description

The genus *Rauvolfia* is pantropical in distribution and contains a variety of alkaloids. The genus includes two very important medicinal species viz. *Rauvolfia serpentina* (L.) Benth. ex Kurz (an Indian species commonly known as sarpagandha in India) and *R. tetraphylla*. Both the species contained bioactive alkaloids having certain biological activities. It belongs to the family Apocynaceae and encompasses herbs or shrubs with leaves in whorls of 3 or 4 (Mahalakshmi *et al.* 2019).

The plant has a straight, smooth, and greyish-brown bark. The branches are opposite, quadrangular, and glabrous. The leaves are simple, opposite, and decussate, meaning that each successive pair is at a right angle to the previous pair. The leaves are elliptic to ovate in shape, measuring 5-15 cm long and 3-7 cm wide. They are dark green on the upper surface and lighter green on the lower surface, with a glossy texture. The leaf base is acute to

rounded, while the tip is acute to acuminate. The leaf margin is entire, and the midrib is prominent on the lower surface. The petiole is 1-2 cm long and is often twisted at the base (IMPPAT | IMPPAT: Indian Medicinal Plants, Phytochemistry and Therapeutics (imsc.res.in)).

The inflorescence is a terminal or axillary cyme, consisting of 2-6 flowers. The flowers are bisexual, fragrant, and tubular in shape, measuring 1.5-2 cm long and 1 cm wide. They are white or pinkish white in colour and have five petals that are fused at the base to form a tube. The calyx is small and five-lobed, while the corolla is funnel-shaped with five lobes. The stamens are inserted near the base of the corolla tube, and the ovary is superior with two carpels. The fruit is a two-celled, oblong or ovoid berry, measuring 1.5-2.5 cm long and 1-1.5 cm wide. It is green when young, turning to yellow or orange when ripe. The fruit contains numerous seeds that are flat, circular, and brown in colour.

The structure and development of drupe fruit of *R. tetraphylla* were analysed by Thomas and Dave (1990). Different developmental stages of fruit were observed, and photomicrographs were taken with Carl-Zeiss microscopes. The fruit of *R. tetraphylla* are small glabrous drupe, developing from bicarpellary syncarpous superior ovary with ovules on its axile placentation. At the extreme base both the carpel is nearly free but towards the upper half of the ovary both the carpel is entirely fused. Ovary wall is homogenous parenchymatous differentiated into outer epidermis, mesoderm, and inner epidermis. Enlargement of fruit is initiated after anthesis; to become a drupe but rarely both the carpels separate incompletely and enlarge into twin drupe. Ovary wall developed into pericarp with three distinct parts such as epicarp, mesocarp and endocarp. Vascular bundles are arranged more or less in a ring and are extremely differentiated. Towards the maturity of the fruit, cells are enlarged greatly and are well vacuolated. Laticifers are non-articulated types with milky latex. Tanniferous cells or crystals are entirely absent in fruit (Thomas and Dave, 1990). *R. tetraphylla* is a highly valued plant due to its medicinal properties, its attractive foliage and flowers make it an ornamental plant as well.

2.6. General Description of *Rauwolfia* Species

Five different species such as *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla* and *R. verticillate* were analysed the morphological character of seeds and fruits which occur in South India. Fruit of *R. hookeri* contain semi-apocarpus type which are glabrous, ovoid in shape, when young cream-yellow in colour, dark purple in colour at ripen. *R. micrantha* of fruit consist of semiapocarpous, glabrous, ovoid, yellowish green in colour at young stage,

when ripen it become dark purple in colour. *R. serpentina* fruit contain glabrous, semi-apocarpous, dumbbell shape, when young it is dark green, and it became dark purple during ripen. *R. tetraphylla* fruit consist of pubescent texture, syncarpous type, globose in shape, reddish in color at young stage and dark purple at ripen stage. Fruit of *R. verticillate* contain glabrous texture, dark purple in colour when ripen and dark green during young, ellipsoid in shape and apocarpous type. Seeds of the five different species of *Rauvolfia* exhibit variations with respect to hilar groove and presence or absence of wax. Three of the species show specific hilum features except *R. hookeri* and *R. micrantha*. The five different species of *Rauvolfia* can be segregated based on the characters of fruits and seeds and helps to assist identification of species (Kumar *et al.* 2011).

2.7. Traditional Medicinal Uses

Rauvolfia tetraphylla holds immense medicinal value and is widely used by South Indian tribes. The various parts of the plant are used to treat a wide range of ailments. The roots of *R. tetraphylla* are used to stimulate uterine contractions during difficult deliveries, while the shoots are used to alleviate stomach pain. The root extract is also used to treat muscular and rheumatic arthritis, and the leaf decoction is used to relieve cough and cold symptoms. The leaf paste is applied to the skin to treat skin diseases, while the root paste, when consumed twice a day on an empty stomach for 21 days, is believed to help in curing mental disorders. *R. tetraphylla* is also known to possess potent antihypertensive properties and is commonly used to treat high blood pressure. The powdered root of the plant is effective against ulcers, worms, and when applied externally, can help alleviate chronic skin diseases. The leaf extract is also widely used to treat various ailments such as anxiety, epilepsy, and nervous disorders. Apart from these, *R. tetraphylla* is also known to be an antidote for snake bites, insect stings, and animal bites. It is also used to treat insomnia, high blood pressure, and madness. The leaves and flowers of the plant are consumed to treat asthma (Kalam Khan, 2013). It is a highly versatile plant that possesses numerous medicinal properties and has been widely used in traditional medicine for centuries.

2.8. Ethnobotany

Traditional medicine is the primary choice of disease treatment for many people living in remote areas, particularly ethnic groups (Mbuni *et al.* 2020). In the Bodoland region of Assam, tribal communities have been using medicinal plants, such as *Cassia fistula*, *Lindernia crustacea*, *R. tetraphylla*, *Ricinus communis*, *Sacciolepis myosuroides*, and

Zingiber zerumbet, as deworming agents and for treating various diseases (Uniyal *et al.* 2006).

Rauvolfia serpentina is a flowering plant in the Apocynaceae family and has been traditionally used in Ayurveda to treat various diseases such as insanity, epilepsy, insomnia, hysteria, eclampsia, and hypertension. Its root gained popularity in the 1950s for its anti-hypertensive effects attributed to the alkaloid found in the root. Ayurveda prefers the use of whole herb over extracts for better efficacy, enhanced bioavailability, reduced side effects, smooth excretion, and prevention of drug resistance. Reserpine is an extract from Sarpgandha, has reported many adverse drug reactions and drug resistance, leading to discontinuation in hypertension management (Bunkar, 2017).

Rauvolfia serpentina has been used in Ayurvedic medicine for centuries to treat various ailments, provides active chemical constituents, and its pharmacological properties with a focus on its potential use in cardiovascular and central nervous system disorders. Alkaloids are the primary constituents of *R. serpentina* and they impart various pharmacological properties. The most active compound in *R. serpentina* is the indole alkaloid reserpine. The plant is also known to possess antimicrobial and antidiarrheal properties, in addition to being used for the treatment of circulatory disorders, rheumatism, hypertension, epilepsy, and insanity. The leaves of the plant are used in the removal of corneal opacities. It indicated that *R. serpentina* is a promising source of compounds with medicinal applications and represents an intriguing subject in the quest for new natural drugs (Singh, 2017).

2.9. Additional Medicinal Properties

Rauvolfia tetraphylla is a member of the Apocynaceae family and has significant ethnobotanical and pharmacological significance in the Indian traditional system of medicine. The plant is widely used by South Indian tribes and various alkaloids have been isolated from it, which possess important pharmacological activities such as antipsychotic, antimicrobial, anti-inflammatory, anticancer, antihypertensive, antidiarrheal, antioxidant, and miscellaneous activities (Iqbal *et al.* 2013).

Epizootic ulcerative diseases (EUD) are one of the dreadful diseases caused by fungal pathogens, *Aphanomyces invadans*, which affect fresh and brackish water feral. Due to these diseases, most carp are affected which is susceptible to the source of aqua protein. The effect of diet supplemented with the *R. tetraphylla* ethanolic extract of leaf against *A. invadans* in Indian major carp, *Labeo rohita* was analysed to find out the innate immune and disease response. The diet is supplemented to infected fish fed with 5g/kg in which WBC,

biochemical parameters, phagocytic and lysozyme activity were carried out which increased after the second week. The respiratory burst activity, complement activity and myeloperoxidase activity were greatly increased by the third week in 5 and 10 g/kg supplementation diet. These diets help to increase the antioxidant activity after the second week. Through this study 5 g/kg diet supplement increase the innate immunity and confer disease resistance help to extend prophylactic strategy for sustainable aquaculture (Yogeshwari *et al.* 2015).

2.10. Seed Germination Vigour and Allelopathy

Effect of different propagating medium and plant growth promoting Rhizobacteria (PGPRs) on seed germination and seedling vigour of *R. tetraphylla*. Eight treatments were observed in Completely Randomized Design (CRD) for four replications in germination percent, germination rate, seedling height, girth, seedling vigour, root length and total biomass. The higher seed germination (16.00%) was observed in the medium containing Red Soil + Sand + Vermicompost + Rhizobium + Phosphate Solubilizing Bacteria (PBS) + *Pseudomonas fluorescens*. The maximum seed height (17.37 cm), seedling girth (0.23 cm), number of leaves per seedling (6.40), leaf area (28.14 cm²), seedling vigour (412.27), total biomass (1.71 g) and root length (6.50) was recorded in media contain Red Soil + Sand + Vermicompost + Rhizobium + PSB + *Pseudomonas fluorescens*. As a result, compared to other media, Red Soil + Sand + Vermicompost + Rhizobium + PSB + *Pseudomonas fluorescens* showed better propagating media composition (Kademani *et al.* 2017).

Allelopathy refers to the advantageous or detrimental effects of one plant on another plant by liberating the chemicals from its components through leaching, root exudation, volatilization, residue decomposition and others in natural and agricultural systems. To appraise the allelopathic action of *R. tetraphylla* touching to the alteration of germination, exaggeration of seedling and biochemical actions in gram seed (*Cicer arietinum* L.). Various concentrations of (12.5, 25, 50, 100, 150, and 200 mg/mL) aqueous extracts were prepared from the root of *R. tetraphylla* and germination studies were conducted. The medium concentrations (50 mg/mL and 100 mg/mL) of root extracts stimulated the seed germination, growth, and biochemical constituents (total sugar, protein, amino acid and DNA and RNA concentrations) of gram. A significant variation is found among all treatments considering seedling parameters and seed treatment is better than control. In biochemical observations, it is the same where T4 (100 mg/mL) is indicating highest performance in fresh and dry weight.

The enhanced action in occurrence of root extract can be supportive for early establishment of seedlings especially in water stress locations (Mandal *et al.* 2013).

The performance of advanced genotypes can be hindered by unhealthy seedlings with low-vigour seeds, which can be improved through seed invigoration. The rhizosphere pattern and seedling performance of *C. arietinum* L. were evaluated by assessing the allelopathic action of *R. tetraphylla* root extracts on seed vigour. Different aqueous root extracts (at concentrations of 200, 150, 100, 50, 25, and 12.5 mg/mL) were prepared and the effects were observed at the 8th and 14th day after overnight soaking of the seeds. Significant variations were found in diverse seedling and root parameters, with the 100 mg/mL extract stimulating total root length and surface area, as well as shoot length and vigour index. The allelopathic action of the root extracts enhanced the quality of the seedlings through modification of the rhizosphere pattern, leading to improved seedling performance compared to the control. It suggested that allelopathic seed invigoration could be considered as a potential method for improving seed vigour and promoting crop production (Mandal & Chakraborti, 2021).

2.11. Plant Tissue Culture

Rauvolfia tetraphylla species contain important medicinal properties due to the presence of various indole alkaloids. Among 30 alkaloids analysed in this species, reserpine was one of the most important compounds present in this plant and greatly helpful in pharmaceutical industries. PTC is one of the alternative techniques to synthesize the value of secondary metabolites. The leaf was taken as an explant and inoculated on MS medium to produce a calli with 2,4-D, NAA, IBA and IAA. From these hormones 9 μ M 2,4-D showed maximum (95%) of callus induction. The calli formed in these PGHs were subjected to NaCl salt treatment with different concentrations (0, 25, 50, 75 and 100 mM). The effect of salinity on callus growth and reserpine accumulation was evaluated. Normal callus growth was found up to 50 μ M concentration of NaCl but the callus growth was reduced at 75 mM and there is no callus growth at 100 mM concentration, it leads to complete cessation. It showed that accumulation of reserpine was increased up to 75 mM NaCl concentration (Anitha and Kumari, 2006).

In mature plants, nodal segments of *R. tetraphylla* were used to initiate *in vitro* cultures. The explants were cultured on MS medium with BA (10 μ M), NAA (0.5 μ M), NAA (0.5 μ M), 3% sucrose and solidified with 0.8% agar. Shoots were developed from the nodal segment and transferred to fresh medium for further culture. For encapsulation, the nodal segments of around 3 mm were dissected aseptically. Different concentrations (1,2,3,4 and

5%) of sodium alginate were prepared using MS liquid medium. Encapsulation was accomplished by mixing the nodal segments into the sodium alginate and dropping into calcium chloride solution. After 30 mins, the beads were formed by polymerization of sodium alginate and rinsed with sterile water to remove the calcium chloride residues. The nodal segment which is encapsulated showed successful regeneration after different periods (1-8 weeks) of cold storage at 4°C. Among the five different periods the beads showed maximum frequency on 4th µM IBA. Root and shoot were developed, and the plantlets were transferred to *ex vitro* condition and to soil. High viability of alginate coated nodal segment after 4 weeks storage is greatly hopeful for the exchange of *R. tetraphylla* genetic resources (Faisal and Mellas, 2002).

Anitha and Ranjitha investigated an *in vitro* culture of *R. tetraphylla*. Nodal segment and shoot tip were inoculated on MS medium with various growth hormones such as BAP and Gibberellic acids (GA₃). The shoot bud production was observed after 5 to 6 days of inoculation. As a result, BAP medium shows maximum shoot production for each explant of about 4.56 ± 0.098 shoots were produced with 4.4 µM concentration of BAP after two weeks of inoculation. The production has ceased when the concentration exceeds 8.88 µM concentration of BAP. Flower bud production and flower induction was observed at 4.44 µM of BAP with 4.3 µM of GA₃. Moreover, the maximum number of shoots were reduced to three in the same concentration. *In vitro* flowering was observed from the terminus of the regenerated shoot tip in the medium consisting of 4.4 µM of BAP with 4.33 µM of GA₃ (Anitha and Ranjitha, 2006).

In vitro shoot proliferation was taking place from the nodal explant of *R. tetraphylla* with the effect of thidiazuron (TDZ). MS medium with TDZ was efficient in inducing the shoot buds. MS basal medium containing different concentrations of TDZ (0.5, 2.5, 5, 7.5 and 10 µM) were used for shoot propagation and development. The maximum (90%) rate of shoot regeneration and its mean number (18.50 ± 1.25) per explant were observed from the nodal segment maintained on MS medium containing 5 µM of TDZ for 4 weeks compared to that of control. The shoot was regenerated whereas root is formed on MS medium with 0.5 µM IBA. The plantlets which are micro propagated were hardened to carry on *ex vitro* conditions. The well-developed plantlets were transferred to sterile vermiculite and enclosed with transparent polyethylene bags to make certain high humidity. After 1 month, plants were transferred in earthen pots holding garden soil and kept in a greenhouse soil. The maximum (90%) survival rate was observed (Faisal *et al.* 2005).

Faisal *et al.* developed a method for plant regeneration and establishment from alginate encapsulated synthetic seeds of *R. serpentina*, a significant medicinal plant. The *in vitro* proliferated micro shoots to produce synthetic seeds through complexation of 3% sodium alginate prepared in Lloyd and McCown woody plant medium (WPM) and 100 mM calcium chloride. The re-growth ability of encapsulated nodal segments was evaluated after storage at 4°C for different periods and compared with non-encapsulated buds. The effects of different media on conversion into plantlets were investigated in which the highest frequency was achieved on WPM with 5.0 µM BA and 1.0 µM NAA. Rooting in plantlets was achieved in a half-strength MS liquid medium containing 0.5 µM IAA. The genetic fidelity of *Rauvolfia* clones raised from synthetic seeds following four weeks of storage at 4°C was assessed using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. The RAPD and ISSR profiles from generated plantlets were monomorphic and comparable to the mother plant, indicating genetic stability among the clones (Faisal *et al.* 2012).

Faisal *et al.* reported a production of encapsulated artificial seeds of *R. tetraphylla* by using sodium alginate and calcium chloride solutions in WPM. After polymerization, the beads were collected and then cultured in different mediums such as MS medium, WPM, B5 and SH (Schenk and Hildebrandt) medium supplemented with 0.3 µM BA and 2.5 µM NAA. Beads with low concentration show fragile and easy to handle contain maximum percentage of growth when compared to high concentration of sodium alginate, which is hard in texture and unsuitable that causes delay in germination. RAPD and ISSR techniques were used to assess the genetic stability between the plantlets and the mother plant, in which total DNA was extracted by Doyle and Doyle (1987) method and amplification takes place. The primer obtained from the plantlet and the mother plant by using RAPD and ISSR-PCR technique show monomorphic pattern, which conforms to the uniformity of genetic fidelity of artificial synthetic seed derived plantlets. This method is used to preserve germplasm and *ex situ* conservation of endangered medicinal plants (Faisal *et al.* 2012).

Rohela *et al.* described a standardized protocol for *in vitro* clonal multiplication using leaf and stem-based callus and assessed the genetic fidelity of acclimated plantlets using start codon targeted (SCoT), inter simple sequence repeats (ISSR), and randomly amplified polymorphic DNA (RAPD) markers. The induced friable callus from leaf, root, and stem explants on MS medium with 2,4-D and NAA. The maximum shoot regeneration from leaf

and stem calluses on MS media with TDZ + BAP and successfully rooted the regenerated shoots on full strength MS media amended with IAA + IBA. After hardening the regenerated plantlets with a sterile garden soil and sand mixture and acclimated them in field conditions with an 86% survival rate. To ensure genetic fidelity, they used SCoT, ISSR, and RAPD primers to conduct PCR analysis comparing the micro propagated plants to the mother plant. S2, R10, and OPA3 primers yielded good amplification with scorable DNA bands. It indicated that the regenerated plants were true-to-type and did not show any polymorphism when compared to the mother plant (Rohela *et al.* 2019).

Alatar & Faisal developed a protocol for producing synseeds of *R. tetraphylla* through *in vitro* microshoot proliferation and encapsulation in sodium alginate and calcium chloride. The effects of different media on the morphogenic response of syn-seeds were evaluated, and the results showed that the highest frequency was achieved on WPM with 6-benzyladenine and NAA. The encapsulated nodal segments stored at 4°C for up to 8 weeks successfully developed into complete plantlets when inoculated on regeneration medium. The resulting plantlets were then established *ex vitro* and were morphologically like the mother plant. Genetic stability of the synseed-derived plantlets was assessed using RAPD and ISSR analysis, which showed no changes compared to the mother plant (Alatar & Faisal, 2012).

Synseeds of *R. tetraphylla* were produced by encapsulating *in vitro*-proliferated shoots using 3% sodium alginate and 100 mM CaCl₂. The encapsulated buds were stored at different temperatures, and the highest conversion rate was observed at 4°C for 4 weeks. The effect of different medium strengths on *in vitro* conversion response of synseeds was evaluated, and the best results were obtained using half-strength WPM supplemented with 7.5 µM BAP and 2.5 µM NAA after 8 weeks of culture. The plantlets were successfully transplanted into field conditions after hardening. After 4 weeks of transfer to *ex vitro* conditions, the performance of synseed-derived plantlets was evaluated and compared to *in vivo*-grown plants based on various physiological and biochemical parameters. The clonal fidelity of synseed-derived plantlets was assessed using rapid amplified polymorphic DNA and ISSR analysis, and compared to the mother plant, with no changes in molecular profiles detected among the regenerated plantlets, confirming genetic stability among clones (Faisal *et al.* 2013).

Hussain *et al.* (2018) investigated the effect of different concentrations of TDZ on *in vitro* bud induction and proliferation of *R. tetraphylla*. The multiple shoots were induced from nodal explants on MS medium with various concentrations of TDZ (0.2–1.0 µM). After 20 weeks of culture on MS medium fortified with 0.4 µM TDZ + 2.0 µM BA, about 30

shoots per explant with an average shoot length of 5.8 cm were obtained, and regeneration frequency was enhanced by 24% compared to TDZ. Microshoots (~5 cm) were successfully rooted on a filter paper bridge in MS liquid medium with different concentrations of IBA after four weeks of incubation. The best rooting response (80%) was achieved on MS medium with 0.3 μM IBA with a mean number of roots of 9.3 ± 1.45 and root length of 3.3 ± 0.33 cm per shootlet. The *in vitro* raised healthy plantlets with well-developed roots and shoots were successfully hardened and acclimatized. The plantlets were successfully established in earthen pots with a 90% survival rate. The genetic fidelity of *in vitro* regenerated plantlets was evaluated using random amplified polymorphic DNA markers, and no polymorphism was observed in the banding pattern among micro-propagated and donor plants, indicating their genetic uniformity and clonal fidelity (Hussain *et al.* 2018).

Rauwolfia serpentina is a plant known for its medicinal properties and is commonly used in western and traditional medicine due to its bioactive compounds such as yohimbine, reserpine, ajmaline, and serpentinine. It exhibited a range of activities including antimicrobial, anti-inflammatory, antiproliferative, and anticholinergic effects. To rapidly multiply this endangered plant, an axenic culture in suspension medium using different combinations of PGHs such as IBA, BAP, KN, NAA, 2,4-D, and TDZ. Maximum shoots were proliferated in liquid MS medium with IBA + KN showed rapid shoot growth, and BAP + IBA + KN was best for increasing the number of shoots. *In-vitro* grown shoots has been successfully rooted and *ex-vitro* in different potting mixtures under greenhouse conditions, achieving a 95% survival rate within 10 days of plantation (Kad *et al.* 2017).

Shahid *et al.* (2016) investigated the effect of heavy metals on the morphogenic response of nodal explants of *R. tetraphylla*. It showed that the addition of copper (Cu), zinc (Zn) and cadmium (Cd) on MS medium had a significant effect on shoot induction. The optimum regeneration was observed on MS medium with (10 μM) BAP, (0.5 μM) NAA, and (100 μM) zinc sulphate, which resulted in 12.2 shoots per explant with a shoot length of 2.5 cm after 12 weeks of culture. The optimum concentration of copper sulphate was found to be 20 μM , resulting in 11.6 shoots per explant and a shoot length of 3.3 cm. *In vitro* microshoots of 3 cm in length were rooted efficiently on 1/2 MS medium with IBA (0.5 μM), which induced 4.8 roots with a root length of 1.8 cm after 4 weeks. The regenerated plantlets were successfully hardened in soil-rite and transferred to garden soil under normal conditions (Shahid *et al.* 2016).

Chang *et al.* (2014) investigated the hairy root culture of *R. verticillata* to express eight genes involved in ajmalicine biosynthesis and the accumulation of ajmalicine in

response to ABA treatment. It showed ABA treatment up-regulated the five MEP pathway genes, while the three ajmalicine-pathway-specific genes did not respond to the treatment. The rate-limiting enzyme gene STR (Strictosidine synthase) showed dramatically decreased expression in the ABA treatment. However, the content of ajmalicine in the ABA-treated hairy roots was significantly higher than in the control, suggesting that the MEP pathway plays an important role in ajmalicine biosynthesis in *R. verticillata*, and STR may not be the rate-limiting enzyme in ajmalicine biosynthesis. It provided valuable insights into the molecular mechanisms of ajmalicine biosynthesis in *R. verticillata* and may have implications for the cultivation and production of ajmalicine (Chang *et al.* 2014).

Harisaranraj *et al.* (2009) analyzed the endangered shrub of *R. tetraphylla* through micropropagation techniques. Explant, nodal segments with two axillary buds were inoculated on MS liquid basal medium supplemented with 0.5 mM kinetin. The explant was subculture and inoculated through which it yields four propagules on each subculture. These cultures were transferred to sterile peat based potting soil for the acclimatization of plantlets. After four weeks the plant was transferred to pot with normal temperature, humidity and micro propagated plantlets were successfully transferred to the greenhouse. *In vitro* plants were transformed to pots containing vermiculite and soil (1:1) and the rate of endurance is 100%. Through this micropropagation technique 150 plants were obtained from 40 explants every 45 days (Harisaranraj *et al.* 2009).

The impact of photosynthetic photon flux density (PPFD) on the antioxidant metabolism and photosynthetic properties in leaves during *ex-vitro* establishment of micro propagated *R. tetraphylla* plantlets were studied. The acclimatization process of *in vitro*-propagated plantlets was carried out under controlled conditions of either low-light (LL) or high-light (HL) conditions, at 50 or 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic PPFD, for four weeks. It showed that the acclimatized plantlets at both light intensities exhibited an increase in chlorophyll a, b, and carotenoid levels. Although micropropagated plantlets were not photosynthetically active at transplantation, the net photosynthetic rate increased over time in newly formed leaves during acclimatization. It suggested that the plantlets adapted from heterotrophic to autotrophic mode of nutrition. The acclimatization process and exposure to different PPFD intensities can significantly affect the growth and development of micropropagated plantlets (Faisal & Anis, 2009).

Anitha & Ranjitha (2006) investigated a stimulation of reserpine biosynthesis in the callus of *R. tetraphylla* by using precursor feeding. Reserpine is a significant alkaloid used to cure hypertension, psychiatric diseases by means of tranquilizing agent, due to which it is in

great demand in pharmaceutical industries. Natural resources of reserpine production are cheaper when compared to chemical synthesis. PTC techniques are one of the enhancing methods to synthesize the reserpine compound. The biosynthesis of reserpine has been initiated by means of tryptophan. The leaf explant was sterilized and inoculated on MS medium with 2,4-D (9 μ M). The effect of tryptophan on callus induction, growth and alkaloids accumulation was determined by adding 25, 50, 75 and 100 mg/L tryptophan in the callus induction medium. The callus was dried and extracted with methanol, and extract was evaporated under vacuum. Crude alkaloids extract was subjected to TLC and reverse phase HPLC by using reserpine as a standard. Accumulation of reserpine alkaloids were observed by addition of tryptophan in the callus induction medium. Compared to other concentrations, reserpine content was increased in 50 mg/L of tryptophan. The cost of that precursor is relative to that of the product (Anitha and Ranjitha, 2006).

Rauvolfia serpentina contains indole alkaloids which is significantly helpful in pharmaceutical purposes. Due to improper way of collection as well as no proper conservation strategy, the plant faces a high threat. So, *in vitro* rapid multiplication using PTC is an effective tool for *ex situ* conservation of this plant species. The seeds of *R. serpentina* were collected and washed under sterilized conditions. The seeds were inoculated on a hormone free MS medium. The different parts of *in vitro* seeds germination were used as an explant such as node, internode, leaves and shoot tip were inoculated on MS medium with various PGRs like IAA, IBA, 2-4-D, KIN and BAP and its combination. All the explants were found to be effective for the induction of callus, but the internodes were less effective than the other explants. The *in vitro* as well as *ex vitro* rooting of the micro shoots have been produced and effectively acclimatized. The best media for shoot multiplication from the node on MS + NAA (0.5 ppm) + BAP (0.5 ppm) and callus cultures on MS + BAP (2.0 ppm). For rooting *in vitro* and *ex vitro*, NAA has been found to be the best among auxins and for callus induction NAA (1.0 ppm) and 2,4-D (2.0 ppm) was effective (Pant and Joshi, 2008).

Nodal segments from *R. tetraphylla* were cultured and encapsulated in calcium alginate hydrogel. The encapsulated buds were evaluated for their morphogenic response to different planting media. The maximum shoot development was observed on MS medium with BA (10 μ M), NAA (0.5 μ M), and 3% sucrose, and gelled with 0.8% agar. The encapsulated nodal segments were successfully regenerated after different periods (1-8 weeks) of cold storage at 4°C. The beads stored for 4 weeks showed the maximum frequency (86.7%) of shoot proliferation when placed on a regeneration medium. The regenerated shoots were rooted on MS medium containing IBA (0.5 μ M). The plantlets were then

hardened to survive *ex vitro* conditions and transferred to soil. The high frequency of plant regrowth from alginate-coated nodal segments coupled with high viability percentage after 4 weeks of storage is encouraging for the exchange of *R. tetraphylla* genetic resources. It demonstrated the potential of using encapsulation technology and cold storage to conserve and propagate *R. tetraphylla* plant material (Faisal *et al.* 2006).

2.12. Phytochemical Analysis

Indole alkaloids have fascinated huge concern from biological and therapeutic agents due to their anticancer, antimalarial, antihypertensive, and sedative properties. Isolation, structure elucidation and cytotoxic evaluation of new compounds has been carried out in *R. tetraphylla*.

Phytochemical analysis has been carried out on the aerial parts of the plant. For isolation of the compound TLC and HPLC with different fractions of elucidation were used. Isolation of five new alkaloids, rauvotetraphyllines A-E, as well as eight known analogues, alstonine, nortetraphyllicine, peraksine, sarpagine, 3-hydroxysarpagine, dihydroperaksine, 10-hydroxydihydroperaksine and raucaffricine were obtained. The new alkaloids were evaluated for their cytotoxicity *in vitro* against five human cancer cell lines (Human Myeloid Leukemia HL-60, Hepatocellular carcinoma SMMC-7721, Lung cancer A-549, Breast cancer MCF-7 and Colon cancer SW-480) by using the MTT method and IC₅₀ value were deliberate by Reed and Muench's method. The structure was recognized by means of spectroscopic methods (UV, IR, NMR, ESIMS and HRESIMS) (Gao *et al.* 2012).

A new labdane diterpene, 3 β -hydroxy-labda-8(17),13(14)-dien-12(15)-olide, was isolated from the air-dried stems and branches of *R. tetraphylla*. This compound was identified through various spectroscopic techniques, including UV, IR, MS, and NMR (¹H, ¹³C, and HMQC). It is the first time that this terpenoid constituent has been reported in *R. tetraphylla*, which is an important medicinal plant known for producing a variety of alkaloids (Brahmachari *et al.* 2011).

Rauvolfia verticillata (Lour.) Baill., also known as Luofumu in Chinese, has been widely used in traditional Chinese medicine for its potential to lower blood pressure. HPLC analysis with UV detection was developed to measure five bioactive indole alkaloids in *Rauvolfia*, including sarpagine, yohimbine, ajmaline, ajmalicine, and reserpine. The HPLC method showed good regressions for all five compounds, and the recovery of the method was found to be in the range of 90.4 – 101.4%. In addition, GC-MS method was developed for the identification of highly volatile compounds in *Rauvolfia* using a DB-1 silica capillary column

(30 m × 0.25 mm i.d., 0.25 μm). More than 39 compounds were identified using the mass function and NIST database (Hong *et al.* 2013).

Rauvolfia serpentina is a valuable medicinal plant with numerous therapeutic properties and it contains alkaloids, carbohydrates, flavonoids, glycosides, phlobatannins, phenols, resins, saponins, sterols, tannins, and terpenes. It contains more than 50 alkaloids belong to the monoterpenoid indole alkaloid family, and the major ones are ajmaline, ajmalicine, ajmalimine, deserpidine, indobine, indobinine, reserpine, reserpiline, rescinnamine, rescinnamidine, serpentine, serpentinine and yohimbine. It exhibited antimicrobial, antifungal, anti-inflammatory, antiproliferative, antidiuretic and anticholinergic activities. Due to its cultural acceptability, compatibility with the human body and low side effects, herbal medicine remains the primary healthcare option for 75 - 80% of the world population. Thus, exploring alternative natural remedies is crucial for curing millions of people globally (Kumari *et al.* 2013).

Hong *et al.* (2012) identified a new acridone alkaloid, called 9-hydroxynoracronycine, in addition to three known compounds including coumarins, lignan, and indole alkaloid, from the roots and rhizomes of *R. verticillata*. The chemical structure of the new compound was determined through spectroscopic methods such as UV, IR, MS, and NMR. It revealed that the acridone alkaloid and coumarins are novel constituents of the *Rauvolfia* genus. Moreover, the cytotoxic effects of the new compound were evaluated against human breast cancer cell line MCF-7 and human promyelocytic leukemia HL-60. It demonstrated that compound significantly inhibited MCF-7 cell proliferation in a dose-dependent manner with an IC₅₀ value of 102.8 μmol/L. It exhibited moderate relaxation effects on the smooth muscle of the small intestine and has potential therapeutic applications in cancer treatment and gastrointestinal disorders (Hong *et al.* 2012).

The chemical composition of two Indian medicinal plants, *R. serpentina* and *Ephedra vulgaris* was analysed, along with their vitamin and mineral content. It showed the presence of various bioactive compounds including alkaloids, saponins, flavonoids, phenols, and tannins. Both plants were found to contain significant amounts of ascorbic acid, riboflavin, thiamine, and niacin, which are important vitamins for human health. Additionally, the plants were found to be rich sources of essential minerals such as calcium, phosphorus, potassium, magnesium, sodium, iron, and zinc. It underscores the significance of these natural sources in providing important nutrients to support human health (Harisaranraj *et al.* 2009).

Rauvolfia species were analysed due to the presence of indole alkaloids, particularly reserpine. The root of Six *Rauvolfia* species were selected such as *R. hookeri*, *R. serpentina*,

R. micrantha, *R. tetraphylla*, *R. verticillata* and *R. vomitoria* were evaluated the reserpine content by using HPLC-ESI-QToF-MS. Through this method reserpine has been identified by exact mass, isotopic peak pattern, tandem mass fragmentation pattern and with authentic standard match. The solvent used for the HPTLC method for the separation of reserpine is hexane: acetone: methanol (6:3.5:0.5, v/v). Among the six species, Reserpine content was highest in exotic species of *R. vomitaria* (689.5 $\mu\text{g/g}$, dry wt.) whereas beside with the five Indian species reserpine content was maximum in *R. tetraphylla* (450.7 $\mu\text{g/g}$, dry wt.) and least amount of reserpine was found in *R. serpentine* (254.8 $\mu\text{g/g}$, dry wt.) and *R. micrantha* (422.1 $\mu\text{g/g}$, dry wt.). It was validated in terms of linearity, specificity, detection limit, quantitation limit, precision, accuracy, and repeatability (Bindu *et al.* 2014).

Gao *et al.* (2015) identified five new hybrid monoterpene indole alkaloids from the aerial parts of *R. tetraphylla* and named as rauvotetraphyllines F-H (1, 3, 4), 17-epi-rauvotetraphylline F (2) and 21-epi-rauvotetraphylline H (5), contain an unusual 2,2-dimethyl-4-oxopiperidin-6-yl moiety. The chemical structures were determined through extensive spectroscopic analysis. *In vitro* cytotoxicity assays were evaluated on the anti-cancer activity of the new alkaloids against five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) using the MTT method. However, all compounds were found to be inactive, with IC_{50} values greater than 40 μM . It emphasized exploration of natural products for the discovery of new compounds with potential therapeutic benefits (Gao *et al.* 2015).

Verma *et al.* (2012) described a HPLC method for the concurrent quantification of three yohimbane alkaloids (YAs) with antipsychotic properties in *R. tetraphylla* leaf extracts. The method allows for the simultaneous determination of α -yohimbine, isoreserpiline and tetrahydroalstonine in the plant, with a limit of detection and quantification of 1.30, 1.16, 1.01 and 4.35, 3.88, 3.39 $\mu\text{g/mL}$ (20 μL injection) for compounds respectively. The samples were analyzed by reverse phase chromatography on a Waters spherisorb ODS2 column using isocratic elution with acetonitrile containing 0.1% TEA and water containing 0.1% TFA (35:65, v/v) at a flow rate of 1 mL/min, a column temperature of 30°C and UV detection at 210 nm. It was validated and applied for quantification of individual alkaloids in leaf extracts of this plant species (Verma *et al.* 2012).

Rauvolfia serpentina were determined the altitudinal variation of stigmasterol, a potential antivenin, in roots from seven populations and analysed by HPTLC, with benzene-acetone as the mobile phase. It demonstrated that stigmasterol was present in *R. serpentina* roots at an R_f value of 0.44 and its content varied in different altitudes. It validated the

method for precision, recovery and demonstrated a simple, rapid, precise, and sensitive protocol for qualitative and quantitative determination of stigmasterol from different populations of *R. serpentina*. The presence of stigmasterol in *R. serpentina* roots may contribute to the popular ethnomedicinal use against snakebite. It provides the chemical variability of *R. serpentina* and its potential for therapeutic applications (Dey *et al.* 2014).

HPTLC method has been developed for the quantification of reserpine and ajmalicine in the roots of *R. serpentina* and *R. tetraphylla* collected from Punjab and Uttarakhand. It involved the use of methanolic extracts of the roots containing the indole alkaloids reserpine and ajmalicine. The extracts are separated using TLC Silica gel 60 F254 plates with toluene: ethyl acetate: formic acid (7:2:1) as the mobile phase. The quantification of reserpine and ajmalicine is performed in the absorption-reflection mode at 268 nm. It has a high recovery rate of 99.3% and 98.7% for reserpine and ajmalicine, respectively. The calibration curves are linear for both compounds in the range of 200-1200 ng. HPTLC densitometry has been used to estimate reserpine and ajmalicine in the root parts of *R. serpentina* and *R. tetraphylla*. It provided an efficient way to measure the content of these important alkaloids in *Rauwolfia* species, which is crucial for ensuring the quality of herbal drugs (Pandey & Dey, 2016).

Gupta *et al.* (2015) examined the flavonoid content present in the leaves of *R. serpentina*, a medicinal plant commonly found in India. Phytochemical analysis was carried out on an alcoholic extract of the plant, leading to the isolation of a flavonoid compound. Chromatographic and spectroscopic techniques were utilized to identify the structure of the isolated compound as 3,3',4',5,7-pentahydroxy flavones, known as Quercetin, which is a flavonoid compound with numerous therapeutic properties (Gupta *et al.* 2015).

Gupta & Gupta (2015) investigated the presence of flavonoids in the methanolic extract of *R. serpentina* leaves. Phytochemical analysis of the extract was performed to identify the presence of flavonoids using a combination of chromatographic and spectroscopic techniques. The results revealed the presence of a flavonoid structure identified as rutin, specifically 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[α -L-rhamnopyranosyl-(1 \rightarrow 6)- β -D-glucopyranosyloxy]-4H-chromen-4-one (Gupta & Gupta. 2015).

Rauwolfia serpentina and *Leucas aspera* revealed that they have similar compositions, with high levels of flavonoids, phenolic compounds, and alkaloids, and trace amounts of tannins. It was conducted to examine the chemical properties used in cancer and snake bite treatments (Bhardwaj & Yadav, 2016).

The methanolic extracts of *R. tetraphylla*, *Phyllanthus niruri*, and *Carissa carandas* were evaluated the phytochemical analysis, revealed the presence of significant secondary

metabolites such as terpenoids, tannins, flavonoids, phenols, and alkaloids. HPTLC analysis of the methanolic extracts from the three plants indicated the presence of eight, eight, and five peaks, respectively. The densitometric scanning of the HPTLC chromatogram revealed that the highest peak area corresponded to the compound with the maximum concentration. (Satyanarayana *et al.* 2018).

Rauwolfia densiflora (wall) Benth. ex Hook.f. also known as "Paarisirunila" to the kanikkars, is an erect herb that contains biologically active compounds such as reserpine, densiflorine, ajmaline, sarpagine, rescinnamine, reserpiline, isoreserpiline, and reserpinine. These alkaloids exhibited various activities such as antimicrobial, anticancer, antihypertensive, and antioxidant effects. It provided information on ethnobotanical, phytochemical, and pharmacological investigations of this plant species (Iqbal *et al.*, 2013).

Pharmacognostic studies of the leaf, stem, and root of *R. tetraphylla* were analyzed, and preliminary phytochemical analysis of various extracts revealed the presence of various phytoconstituents such as alkaloids, flavonoids, tannins, phenols, and saponins. A new labdane diterpene, 3 β -hydroxy-labda-8(17),13(14)-dien-12(15)-olide, was isolated from the stems and branches of *R. tetraphylla*. The leaf contained three antipsychotic indole alkaloids such as α -yohimbine, isoreserpiline, and 10-methoxy tetrahydroalstonine. Furthermore, eight known analogues along with five new indole alkaloids called rauvotetraphylline, were isolated from the aerial parts. From a pharmacological perspective, it exhibited various activities such as antibacterial, antifungal, anti-inflammatory, antioxidant, cytotoxic, cardio tonic, and cardio protective activities. *R. tetraphylla* is an important medicinal herb due to its pharmacognosy, phytochemistry, and pharmacology (Jakaria *et al.* 2016).

Vinay *et al.* (2015) evaluated the phytochemical composition and HPTLC fingerprint profile of the n-hexane extract from the leaf of *R. tetraphylla*. The HPTLC fingerprint profiles were assessed using toluene: ethyl acetate: acetic acid (55:45:1 v/v) as the mobile phase. It displayed several peaks with varying R_f values when visualized at 254 nm and 366 nm. Fourteen peaks were observed at 254 nm, while 10 peaks were observed at 366 nm. HPTLC profile of the leaf extract observed the number of chemical components and their concentration, which is crucial in identifying the constituents of medicinal products, as well as detecting additives in herbal medicine. It obtained from the evaluation of HPTLC fingerprint images can be useful in identifying unknown bioactive compounds with therapeutic efficacy (Vinay *et al.* 2015).

The qualitative analysis of phytochemicals like alkaloids, flavonoids, saponins, tannins, gums and mucilages were evaluated in *R. tetraphylla*. The extracts (Petroleum ether,

Benzene, Chloroform and Methanol) of leaves, stems, roots, and their callus (NaCl stressed and unstressed) of 24-month-old *R. tetraphylla* were assessed and determined their antibacterial and antifungal activities. The phytochemical screening revealed that alkaloids were the major phytochemicals in the extracts of leaves, stems, and roots of *R. tetraphylla*. The methanol-based leaf extracts showed maximum bacterial growth inhibition zone (25.0 ± 2.4 mm) against *Staphylococcus aureus*, while methanol-based root extracts showed maximum fungal growth inhibition zone (22.0 ± 2.1 mm) against *Fusarium oxysporum*. Although the NaCl stressed callus extracts exhibited antibacterial and antifungal activities (Rohela *et al.* 2016).

The leaves of *Rauvolfia canescens* were extracted using ethanol in a Soxhlet extractor to obtain essential metabolites. Qualitative phytochemical analysis and antibacterial activity was evaluated against *Bacillus cereus*, *S. aureus*, *Salmonella typhi*, and *Escherichia coli* using both disc diffusion and well diffusion methods. The extract demonstrated significant antibacterial activity with a zone of inhibition, indicating the presence of bioactive compounds with bactericidal properties. The antioxidant potential of the ethanolic extract was determined using the DPPH assay. GC-MS analysis was performed to identify the bioactive compounds from the chromatographic peak, and these compounds were subjected to *in silico* docking analysis with bacterial life cycle receptors using Autodock 4.2 and Cygwin. It showed that the bioactive compounds had a negative binding energy, indicating their inhibitory effect on the bacterial life cycle receptors (Helan and Vignesh, 2016).

Phytochemical studies, antioxidant and anticancer activity were evaluated by leaf and fruit of *R. tetraphylla*. Solvent such as hexane, chloroform, acetone, and methanol were extracted by Soxhlet extraction, and concentrated using a rotary evaporator. Preliminary phytochemical screening revealed the presence of alkaloids and flavonoids from leaf and fruit samples. Qualitative antioxidant analysis has been taken by TLC based DPPH assay and nitric oxide assay. DPPH radical scavenging activity has taken in which ascorbic acid are used as a standard and Quercetin as a standard for nitric oxide scavenging assay. All the extract showed better antioxidant activity except hexane which showed mild antioxidant activity. Cytotoxic activity was followed by brine shrimp assay. In leaf extract, chloroform shows highest potential whereas in fruit acetone shows maximum potential (Behera *et al.* 2016).

Verma (2017) analysed alkaloids present in methanol extract of *R. tetraphylla* (very young leaf, mature leaf, old leaf, flower, flower stem, fruit, stem and roots). After filtration,

basification, evaporation, the dried residue was obtained and articulated as crude for alkaloids. It was analysed with the help of TLC plates and was seen in UV light. Qualitative detection showed that in root ajmalicine, ajmaline, reserpine, serpentine and yohimbine were present. In mature leaves, reserpine, yohimbine, ajmaline and serpentine were observed. In stem, ajmaline, rescinnamine, reserpine, serpentine and yohimbine were detected. It was experimental that total alkaloids from diverse parts of plants ranged from 0.22 to 9.0% (Verma, 2017).

Rauwolfia species are a valuable source of various chemical compounds, particularly the MIAs. Over 226 MIAs and 30 non-alkaloid compounds have been isolated from *Rauwolfia* species with their biological and medical applications being studied. These compounds exhibited a wide range of biological activities such as antioxidant, anti-inflammatory, anticancer, and antibacterial properties. Synthetic chemists interested in the preparation of these scaffolds in laboratory conditions, particularly the most important MIAs such as reserpine, yohimbine, and ajmalicine. It provided an overview of the isolated chemical compositions of MIAs from *Rauwolfia* species over the past 50 years (Boğa *et al.* 2019).

Desorption electrospray ionization mass spectrometry imaging (DESI-MSI) and UPLC-ESI/MS investigated the spatial localization of various indole alkaloids in developing fruits and plants of *R. tetraphylla*. It showed that ajmalicine, yohimbine, demethyl serpentine, and mitoridine were mainly localized in the fruit coat, while ajmaline was found in the mesocarp of the fruit. In the whole plant, the highest ESI-MS intensities of many of the ions were found in the roots, followed by the shoot region. Within the root tissue, all indole alkaloids, except sarpagine and ajmalicine, were found in the epidermal and cortex tissues. In leaves, only serpentine, ajmalicine, reserpiline, and yohimbine were observed, while serpentine being restricted to the petiolar region. Principal component analysis based on the presence of indole alkaloids distinguished the four tissues (stem, leaves, root, and fruits) into distinct clusters. It provided the possible pathways of indole alkaloid biosynthesis and translocation during fruit and seedling development in *R. tetraphylla*, as well as the functional significance of the spatial patterns in the distribution of metabolites (Kumara *et al.* 2019).

The preliminary phytochemical analysis of *R. tetraphylla* revealed the presence of flavonoids, terpenoids, phenols and is well-known to produce MIAs (Lezin *et al.* 2024). The ethyl acetate extract was found to contain over 20 compounds through GC-MS analysis. The silver nanoparticles synthesis was confirmed through UV spectra around 440nm, and

characterization revealed their unique properties using FTIR and SEM. It suggested that the ethyl acetate extract of *R. tetraphylla* leaves possesses great pharmacological properties and potential for drug formulation (Krishnasamy *et al.* 2023).

The pharmacognostical and phytochemical studies analysed on the root of vanasarpagandha (*R. tetraphylla*). The root possessed several diagnostic characteristics such as longitudinal fissures on the outer surface, a greyish brown to reddish brown outer surface, compound starch grains, prismatic crystals of calcium oxalate in the cortex region, uni- to biseriate medullary rays filled with starch grains, xylem vessels with pitted thickenings, and elongated stone cells with broad lumen. In addition to its diagnostic characteristics, the root of *R. tetraphylla* contains several bioactive compounds including alkaloids, steroids, tannins, and carbohydrates. TLC revealed the presence of many bioactive compounds in the root. It suggested that *R. tetraphylla* has potential for use in traditional medicine, as it contains a variety of beneficial compounds (Prathapa *et al.* 2020).

B- Glucosidase assay and protein estimation were evaluated from different parts of *R. tetraphylla* such as fruit, flower, flower stem, very young leaf, mature leaf-1 and 2, old leaf, stem and root. Protein estimation was performed colorimetrically with BSA as standard using Lowry's method. The activity of β - Glucosidase enzyme was maximum in very young leaf (100%), followed by mature leaf-1 (87.16%) whereas lowest in old leaf (15.0%). The highest soluble protein content was found in mature leaf-1 (9.241 mg/mL) which was followed by very young leaf (9.216 mg/mL). Root (2.158 mg/mL) contains lowest activity of protein estimation. From native gel developed of crude enzyme, it has three isoforms found in flower stem, very young leaf and mature leaf-1 and played a significant role in ajmaline and vomiline biosynthesis (Singh & Verma, 2017).

Paul & Xavier (2020) investigated the phytochemistry and antioxidant properties of five members of the Apocynaceae family, namely *Catharanthus roseus*, *Plumeria alba*, *Pentalinon luteum*, *R. tetraphylla*, and *Nerium oleander*. Methanolic extracts of the plants were subjected to preliminary phytochemical analysis for protein, carbohydrate, alkaloids, phenol, tannin, flavonoids, and saponin. The quantification of these components was carried out using spectrophotometry with different standards and methods. The antioxidant properties were evaluated by DPPH using the Blois method with modifications. The results indicated that *R. tetraphylla* had the highest protein concentration, while *N. oleander* had the lowest. *Catharanthus roseus* exhibited the highest concentration of carbohydrate and tannin, while *N. oleander* had the lowest. *C. roseus*, *P. alba*, and *R. tetraphylla* had the highest concentrations of alkaloids, phenol, and flavonoids, while *N. oleander* had the lowest. Furthermore,

N. oleander showed the highest scavenging properties, indicating a high antioxidant activity. It suggested that the plants possessed significant phytochemical and antioxidant properties, which could have potential applications in the development of natural products (Paul & Xavier, 2020).

CT *et al.* (2020) analysed the phytochemistry and biological activities of *Rauvolfia* species such as *R. tetraphylla*, *R. hookeri*, *R. micrantha*, and *R. verticillata*, were evaluated. It found that the root of *R. tetraphylla* has a similar phytochemical profile to that of *Rauvolfia* species, as evidenced by HPTLC analysis. LC/MS characterization also revealed that many of the active alkaloids are common to both *R. serpentina* and *R. tetraphylla*. The anti-hypertensive and analgesic activities of these species were evaluated in experimental animal models and showed significant analgesic activity. It was evidenced that the root of *R. tetraphylla* could serve as a validated substitute for Sarpagandha and may be used as an alternative to the critically endangered *R. serpentina* in Ayurvedic medicine (CT *et al.* 2020).

2.13. Bioactive Compounds and Pharmacognosy

The bioactive compounds were analysed in *R. tetraphylla* and *Shorea robusta*, revealing the presence of alkaloids, flavonoids, phenolics, tannin, glycosidase, reducing sugars and terpenoids. It exhibited antimicrobial activity against human pathogens such as *S. aureus* and *Klebsiella pneumonia*. Additionally, the antioxidant activity of the plants was evaluated using DPPH free radical scavenging assay, with *R. tetraphylla* demonstrating higher efficiency (53.22%) than *S. robusta*. *R. tetraphylla* methanolic leaf extracts were subjected to Alpha-amylase inhibition assay, and showed 87.47% inhibition, while *S. robusta* had a lower inhibition potential of 44.93%. It suggested that *R. tetraphylla* could be useful for protecting cells from oxidative damage (Archana & Jeyamanikandan, 2015).

The cardio protective activity and *in vitro* phyopharmacological effect of *R. tetraphylla* were analysed, in which five groups with six rats have been used for experiment. The Myocardial Infarction (MI) was induced in an experiment by intraperitoneal injection of isoproterenol hydrochloride 20 mg/ 100g body weight, dissolved in physiological saline for two successive days. The ethanolic leaf extract of this plant is combined with the isoproterenol (ISO) hydrochloride used in each group of rats. The pretreatment of leaf extract improved cardiac functions, the effect which is distributed by ISO challenge, through restoration of endogenous antioxidants, controlling lipid peroxide formation and preserving cardiac marker enzyme activities of creatine kinase (CK), lactate dehydrogenase (LD),

activity of SGOT (AST) and activity of SGPT (ALT). Due to the presence of phytochemicals as phenolic groups that might directly or indirectly involve cardioprotective effects. MI is one of the important causes of death from cardiovascular diseases, the leaf extract of *R. tetraphylla* has a potential to cure heart diseases (Nandhini and Stella Bai, 2015).

Rauvolfia reflexa is a plant belonging to the Apocynaceae family, which has been traditionally used for treating age-related brain disorders. Two indole alkaloids [rauvolfine C (1) and 3-methyl-10,11-dimethoxy-6-methoxycarbonyl- β -carboline (2)] and five known compounds [mascusine B (3), vinorine (4), undulifoline (5), isoreserpiline (6) and rescinnamine (7)] were isolated from the plant's bark. Cholinesterase inhibitory assays and molecular docking were performed to understand the compounds inhibitory activity and molecular interactions. The compounds demonstrated good to moderate cholinesterase inhibitory activity, with IC₅₀ values ranging from 8.06 to 73.23 μ M. Compound 7 was found to be the most potent inhibitor of both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Compounds 1, 2, 5, and 6 were selective towards BChE, while compounds 3, 4, and 7 were dual inhibitors with almost equal inhibitory activity on both AChE and BChE. Molecular docking analysis revealed that compounds 6 and 7 interacted differently on AChE and BChE, through hydrophobic interactions and hydrogen bonding. In AChE, the indole moiety of both compounds interacted with residues lining the peripheral anionic site, while in BChE, their methoxy groups primarily interacted with residues at the enzyme's active site. Compounds 6 and 7 showed the most potent cholinesterase inhibitory activity and may hold for drug development (Fadaeinasab *et al.* 2015).

Gao *et al.* (2015) reported that *R. verticillata* stems were the source of 5 novel hexacyclic monoterpene indole alkaloids such as rauvovertine A (1), 17-epi-rauvovertine A (2), rauvovertine B (3), 17-epi-rauvovertine B (4), and rauvovertine C (5). As well, 17 previously known analogues were also isolated and characterized. The presence of rapid hemiacetal tautomerism in solution resulted in compounds 1/2 and 3/4 being obtained as mixtures of C-17 epimers. The structures of the new compounds were determined through spectroscopic analysis and molecular modelling, with the relative configuration of each epimer established based on computer-generated 3D drawings with minimized energy. *In vitro* cytotoxicity assays against various human tumour cell lines showed that one of the new alkaloids, compound 5, exhibited moderate cytotoxicity with IC₅₀ values ranging from 10.76 to 15.70 μ M, while the remaining new compounds showed no significant cytotoxicity. It suggested the potential of *R. verticillata* as a source of bioactive compounds with potential pharmaceutical applications (Gao *et al.* 2015).

2.14. Antibacterial Activity

The antimicrobial and antifungal activity of *R. tetraphylla* were tested in ethanol extract of leaf against *E. coli*, *Streptococcus lactis*, *Enterobacter aerogenes*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa* and *Proteus vulgaris* using disc diffusion method. The zone of inhibition around the disc was observed, among the bacterial *E. coli*, *E. aerogenes* and *A. faecalis* are more sensitive to crude extract compared to other bacteria. Based on the concentration of extract the zone of inhibition are also increased. The antifungal activity was determined against *F. oxysporum*, *Alternaria helianthii*, *Curvularia lunata*, *Aspergillus niger* and *Penicillium spp.* Among different fungi *A. niger* and *Penicillium spp.* were more sensitive as compared to other fungi. Thus *R. tetraphylla* shows innumerable antimicrobial compounds (Suresh *et al.* 2008).

The antibacterial activity was evaluated in *R. tetraphylla* extracts against eight human species including *S. typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *P. vulgaris*, *P. aeruginosa*, *Klebsiella mobilis*, *E. coli* and *S. aureus*. Three different solvents were used such as petroleum ether, chloroform, and ethyl acetate at different concentrations such as 50 μ L, 100 μ L and 150 μ L. Among the three extracts ethyl acetate exhibited more antibacterial activity which is most effective against *Salmonella paratyphi* which showed 26 mm of maximum inhibition zone and showed inhibitory action against eight pathogens tested. Dry fruit extract of *R. tetraphylla* possessed potential antimicrobial activity which is maximum against *S. paratyphi A*, *S. typhi*, *S. paratyphi B* and *P. aeruginosa*. All the extracts possessed antibacterial activity in which ethyl acetate is more effective (Alagesaboopathi, 2009). Antibacterial assay demonstrated zone formation against the pathogen *S. typhi* and *Shigella dysenteriae* (Naz *et al.* 2022).

Rao *et al.* investigated antibacterial activity of various extracts (Hydro-alcoholic, methanolic, ethyl acetate, and hexane) of *R. tetraphylla* root bark. The cylinder plate assay was evaluated against four Gram-positive (*Streptococcus pneumoniae*, *S. aureus*, *B. cereus*, *Bacillus pumilis*) and four Gram-negative (*E. coli*, *E. aerogenes*, *P. aeruginosa*, *Streptomyces marienensis*) bacteria. It indicated that all extracts of *R. tetraphylla* root bark exhibited a substantial zone of inhibition against the tested bacterial strains (Rao *et al.* 2012).

The antimicrobial activity of *R. tetraphylla* was evaluated using aqueous and methanolic extracts against six bacteria, such as three Gram-positive bacteria (*S. aureus*, *Streptococcus pyogenes* and *Leuconostoc lactis*) and three Gram-negative bacteria (*E. coli*,

P. aeruginosa and *S. typhi*), and four fungi (*A. niger*, *Aspergillus flavus*, *Rhizopus indicus* and *Mucor indicus*) using well diffusion method. The methanolic extract of *R. tetraphylla* exhibited the highest activity in *S. pyogenes* and was effective against all bacterial strains tested, except for *S. aureus* and *E. coli*. The aqueous and methanol extracts were ineffective in inhibiting fungal growth (Kavitha *et al.* 2012).

The plant species which belong to Lamiaceae and Apocynaceae were analysed for antimicrobial activity. Eight species (*Mentha arvensis* L., *Anisomeles malabarica* L., *Hyptis suaveolens* L., *Ocimum gratissimum* L., *Ocimum sanctum* L., *Leucas aspera* (Willd.), *Coleus aromaticus* Benth. and *Ocimum basicilicum* L.) of Lamiaceae and seven species (*C. roseus* (L.) G.Don, *R. tetraphylla* L., *N. oleander* L., *Tabernaemontana divaricata* (L.) R. Br. ex Roem. & Schult, *Allamanda cathartica* L., *Thevetia peruviana* Merr. and *P. alba* L.) of Apocynaceae were selected, totally fifteen species of methanolic leaf extracts were evaluated against the common fish pathogen *Aeromonas hydrophila*. It can be carried out with disc diffusion assay on Mueller Hinton Agar medium. In Lamiaceae, *C. aromaticus* with MIC of 12.5 mg/mL with the inhibition zone of 10.33 mm and in apocynaceae *T. divaricate* MIC value of 12.5 mg/mL with the zone of inhibition of 7.33 mm showed very effective against *A. hydrophila*. *M. arvensis* showed an inhibition zone of 9.67 mm and *L. aspera* exhibited 9.33 mm. In Apocynaceae, *C. roseus* at 25 mg/mL contained a zone of 9.67 mm and *R. tetraphylla* at 50 mg/mL exhibited an inhibition zone of 9.33 mm (Haniffa and Kavitha, 2012).

The phytochemical screening and antibacterial activity were analysed in aqueous, alcoholic and chloroform extracts of leaves of *R. tetraphylla* and *Jatropha curcas* L. against various gram positive and gram negative bacteria such as *B. cereus*, *Bacillus subtilis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *K. pneumonia*, *P. vulgaris*, *P. aeruginosa*, *Streptococcus agalactiae*, *Lactobacillus acidophilus* (ATCC-11975) and *E. coli* by disc diffusion assay with standard antibacterial discs like gentamicin, ampicillin, ceftriaxone, moxifloxacin, penicillin, tetracycline. Methanol solvent of both the plant extract showed better and comparable antimicrobial activity against most of the bacteria except *S. typhimurium* and *E. coli*. Chloroform extracts of *R. tetraphylla* was effective against all the bacteria (12.00±0.58 to 14.67±0.88 mm) but in *J. curcas* had activity against *S. aureus*, *K. pneumonia* and *P. vulgaris* with the zone of inhibition (12.67±1.20 to 14.33±2.03 mm). Water extracts of both plants did not show any antibacterial activity. Alkaloids, tannins, flavonoids, saponins, diterpenes, triterpenes and carbohydrates were observed in the methanol extracts of both the plants by using phytochemical screening (Patel *et al.*, 2013).

Gorai *et al.* (2013) investigated the antibacterial activity of cold ethanol extracts from *Curculigo orchioides* (Amaryllidaceae), *Curcuma longa* (Zingiberaceae), *Limnophila indica* (Scrophulariaceae), *Limnophila rugosa* (Scrophulariaceae), *R. tetraphylla* (Apocynaceae), and *Peltophorum pterocarpum* (Fabaceae) against both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria at 100 mg/mL by disc diffusion method with standard Cefuroxime. It revealed that all plant extracts exhibited higher antibacterial activity against both bacteria than the standard. Among the plants tested, *Curcuma longa* exhibited the highest antimicrobial activity (Gorai *et al.* 2013).

Rauvolfia serpentina is widely recognized for its antibacterial, antifungal, anti-inflammatory, and antiproliferative properties. Reserpine is one of the indole alkaloids present in this plant which were greatly helpful in pharmacological purposes. HPTLC determined the presence of reserpine in both the samples collected from Gadarpur and Uttarakashi. The reserpine was highest (0.37%) in samples collected from Gadarpur when compared to Uttarakashi (0.31%). Negi *et al.* evaluated antibacterial activity by using methanolic root extracts of *R. serpentina* against *S. typhimurium*, *E. coli*, *Citrobacter freundii*, *P. vulgaris*, *Enterococcus faecalis* and *S. aureus* by agar well diffusion method. The maximum zone of inhibition (13 mm) with minimum MIC (625 µg) against *S. aureus* and maximum MIC (10 mg) was observed against *E. coli*. It revealed that it contains a fine quantity of reserpine and strong antibacterial activity against most of the human pathogens (Negi *et al.* 2014).

Alshahrani *et al.* studied the efficacy of *R. serpentina* aqueous leaf extract (RSALE) and gold nanoparticle-encapsulated *R. serpentina* (R-AuNPs) in terms of their antibacterial, antioxidant, and anti-cancer activity. The R-AuNPs are uniform in size, stable, and characterized by various parameters, including UV, TEM, dynamic light scattering, and zeta-potential. The antibacterial activity of RSALE and R-AuNPs was tested via disc diffusion against various gram-positive and gram-negative bacterial, with RSALE (100 mg/mL) showed maximum inhibition compared to R-AuNPs against *S. aureus*, *E. coli*, *B. subtilis*, and *S. pyogenes*, respectively. No zone of inhibition was observed against *P. vulgaris*. MIC values for the tested strains were lower for R-AuNPs than RSALE and Amoxicillin (control), indicating that R-AuNPs have greater antibacterial activity than RSALE. Furthermore, R-AuNPs exhibited higher antioxidant potential than RSALE according to the DPPH and FRAP assays. R-AuNPs showed maximum anti-cancer activity compared to RSALE against the human cervical cancer (Hela) cell line (Alshahrani *et al.* 2021).

Nandhini & Bai (2014) investigated the phytochemical constituents, trace metal concentration and antimicrobial activity of ethanolic extracts from *in-vitro* cultured plant of *R. tetraphylla*. It was inoculated on MS medium with NAA (0.1 mM) + BAP (0.25 mM), showed effective shoot bud induction from nodal explants. Phytochemical analysis revealed the presence of steroids, reducing sugars, sugars, alkaloids, phenols, flavonoids, saponins, tannins and amino acids. HPTLC coupled with mass spectrum analysis identified major compounds such as 3-isoreserpine, ajmalicine, ajmaline, reserpine and yohimbine. Trace metal analysis of the plant samples showed concentrations of Cd, Cr, Cu, Fe, Ni, Pb and Zn as BDL (Below Detection Limits). The ethanol extracts of *in-vitro* *R. tetraphylla* demonstrated significant antimicrobial activity against bacteria and fungi (Nandhini & Bai, 2014).

The antimicrobial potential of leaf and callus extracts of *R. tetraphylla* and *Physalis minima* against selected pathogenic fungi (*Aspergillus ochraceous*, *Aspergillus flavipes*, *Fusarium verticilloides* and *Penicillium* sp) and bacteria (*B. subtilis*, *E. coli*, *Pseudomonas solanacearum*, *Xanthomonas axonopodis* pv. *Malvacearum* and *Xanthomonas vesicatoria*) were investigated using broth dilution assay. Various solvents such as absolute alcohol, benzene, chloroform, methanol, and petroleum ether, were used to extract the leaves and calli. The chloroform extracts of both plants were effective against pathogenic bacteria and fungi, with MIC ranging between 0.25 to 6 mg/mL. Absolute alcohol extracts showed MICs of 0.25 to 4 mg/mL for bacteria and 0.25 to 100 mg/mL for fungi. Extracts obtained from benzene and petroleum ether were ineffective in inhibiting the growth of microbes. The methanol extract showed MICs of 0.25 to 100 mg/mL against bacterial pathogens and 0.5 to 100 mg/mL against fungal pathogens. It suggested that the chloroform extracts of *R. tetraphylla*, *P. minima* leaves and calli have potential as antimicrobial agents against selected pathogenic microbes (Shariff *et al.* 2006).

2.15. Antioxidant Activity

Antioxidant activity and polyphenolic contents were evaluated using three medicinal plant species such as *R. tetraphylla*, *Solena umplexicaulis*, *Rhinacanthus nasutus*. Methanolic extract of leaves were analysed the total phenolic content, flavonoids, antihemolytic activity of H₂O₂ induced red blood cells, ferric reducing power capacity in the range of 10.2 to 25.8 mg quercetin equivalent/g of dry weight sample and phenol content varied from 25 to 41.7 mg gallic acid equivalent/g dry weight sample. Antioxidants were analyzed by using IC₅₀

values. The DPPH activity of IC₅₀ values of *R. tetraphylla*, *R. nasutus* and *S. amplexicaulis* leaf extracts were 170, 230, 190 µg/mL, correspondingly. Whereas the IC₅₀ value of ABTS scavenging activity ranges from 80- 120 µg/mL and antihemolytic activity ranges from 135- 157 µg/mL. In reducing power activity, the IC₅₀ value of *R. tetraphylla* extract was 200 µg/mL as *R. nasutus* and *S. amplexicaulis* extract at a concentration 250 µg/mL did not reach 50% of reducing power. Linear regression was evaluated for antihelmolytic, DPPH and reducing power activities showed a positive correlation. No correlation was obtained between ABTS activity and polyphenol content. Therefore, methanolic extract of plants exhibited the antioxidant activity to some degree of *in vitro* assay (Maheshu *et al.* 2010).

The antioxidant potential, phytochemicals, and nutrient composition were analysed from leaves of five different *Rauvolfia* species (*R. serpentina*, *R. beddomei*, *R. micrantha*, *R. densiflora* and *R. tetraphylla*). Antioxidant activity was assessed using various *in vitro* methods such as DPPH radical scavenging activity, reducing power and superoxide anion scavenging activity at different concentrations. The nutrient and phytochemical composition was determined using spectrophotometric techniques, including HPLC-fluorescence for tocopherols. It showed that *R. serpentina* had the highest total phenolic content, DPPH radical scavenging activity, pigment composition and vitamin E content. *R. tetraphylla* exhibited the highest flavonoid content and β-carotene concentration, while *R. densiflora* had the highest level of vitamin C content and metal chelating activity among the five species. Lycopene was found in very low amounts in all the species, with *R. tetraphylla* having the highest concentration and *R. beddomei* having the least. It suggests that the leaves of *Rauvolfia* species could be a valuable source of natural antioxidants for various industries (Nair *et al.* 2012).

The antioxidant activities were measured using various assays, and their anthelmintic activity against amphistomes. The extracts of *C. fistula*, *R. communis*, and *Z. zerumbet* exhibited high antioxidant properties, and all the plant extracts showed dose-dependent anthelmintic activity against amphistomes. *C. fistula* and *L. crustacea* were found to have the most potent anthelmintic activity, which was comparable to that of the reference drug albendazole. At an equal concentration of 10 mg/mL for all the plants, the mortality rates of amphistomes were found to be highest for *C. fistula* and *L. crustacea*, at 82% and 85%, respectively. It found a positive correlation between the anthelmintic and antioxidant properties of the plants ($p = 0.05$ level) (Swargiary & Roy, 2021). Antioxidant properties were measured using DPPH, and antibacterial activity was assessed using the disk diffusion method. GC-MS analysis was performed to identify the compounds in the extract (Chaves *et*

al. 2020). In addition, silver nanoparticles were synthesized and characterized using FTIR, UV spectrometry, and SEM (Almatroudi, 2020).

The *in vitro* sun protection factor (SPF) of the methanol leaf extract of *R. tetraphylla* was determined using UV and Mansur equation. The SPF value of *R. tetraphylla* was found to be 19.27 at a concentration of 0.4 mg/mL, which is comparable to commercially available sunscreens (Dermatone). The plant extract was also found to contain various phytochemicals such as phenols, carbohydrates, alkaloids, saponins, flavonoids, quinones, steroids, and terpenoids. These compounds are known to have antioxidant and anti-inflammatory properties, which may contribute to the plant's sun protective activity. As concerns grow about the side effects of artificial sunscreens, there is a growing demand for natural and safe alternatives. It suggested that *R. tetraphylla* has potential as a natural source of sun protection and could be developed into a topical sunscreen (Merlin *et al.* 2020).

Oza & Solanki (2016) evaluated the antioxidant activity and total flavonoid content from methanolic extract of fruit from *R. tetraphylla*, *Nyctanthus arbortristis*, and *Calotropis gigantea*. It showed *N. arbortristis* exhibited the highest antioxidant activity (95.96%), followed by *R. tetraphylla* (93.40%) and *C. gigantea* (58.54%). The total flavonoid content of the extracts ranged from 13.2 to 52.7 mg quercetin equivalent/g. It provided an antioxidant potential of these medicinal plants, and as a source of natural antioxidants in the food and pharmaceutical industries (Oza & Solanki, 2016).

Vijay *et al.* (2016) analysed total antioxidant activity from leaves and fruits of n-hexane, dichloromethane, and methanol extracts from *R. tetraphylla* at different concentrations (5, 50, and 100 µg). Butylated hydroxy anisole (BHA) was utilized as a reference standard. It showed that the leaf n-hexane and methanol extracts were significantly active at 5 µg and even more active at 50 µg, compared to BHA. The fruit methanol extract was also found to be active, with its activity increasing with higher doses, though not as potent as BHA. The antioxidant activity of the extracts increases with increasing dose which led to slow down the development of diseases related to oxidative stress (Vijay *et al.* 2016).

2.16. Medicinal and Anticancer Properties

Rauvolfia Serpentina is a tropical shrub with a long history of medicinal use in Indian and Chinese traditional therapeutics. The plant contains various phytochemicals that are responsible for its therapeutic properties. The alkaloids found in *R. Serpentina* have been

extensively studied and authenticated for their pharmacological actions, including lowering blood pressure and sedative effects. Clinical trials have shown its efficacy in treating various neuropsychiatric, gynaecological, and geriatric disorders. It has also been used to treat diarrhoea, dysentery, and gastrointestinal motility disorders, as well as stimulate uterine contractions during childbirth. It suggested the importance of *R. Serpentina* in traditional medicine and emphasizes the need of its phytochemicals for potential therapeutic applications (Agrawal, 2019).

Abuzenadah *et al.* discussed the role of angiogenesis in tumorigenesis and highlighted the importance of targeting the vascular epithelial growth factor (VEGF) and its receptor VEGFR-2 for cancer treatment. It focused on identifying bioactive compounds from the medicinal herb *R. serpentina* that can effectively bind with VEGFR-2. The screened the physicochemical properties of bioactive compounds from *R. serpentina* using the Data Warrior program (version 5.5.0) and selected 17 compounds that met Lipinski's rule of five and showed good drug-likeness for molecular docking studies. The results of molecular docking showed that ajmalicine, 1, 2-dihydrovomilenine, rauwolscine, yohimbine, ajmaline, and papaverine had a strong interaction with VEGFR-2. It concluded that the compounds from *R. serpentina* have the potential to be developed into antiangiogenic drug candidates due to their favourable drug-like properties and lack of significant toxicity (Abuzenadah *et al.* 2022).

Shah *et al.* (2020) investigated the potential antihypertensive and antihyperlipidemic effects of *R. serpentina* in albino rats. It included 30 rats divided into 5 groups, with one group serving as a normal control, one group receiving a high salt diet, one group receiving a standard drug control, and two groups receiving different doses of methanolic plant extract along with a high salt diet. It showed that *R. serpentina* significantly decreased blood pressure and had a positive impact on serum lipid profile and serum proteins. It found that *R. serpentina* had a hepatoprotective and renoprotective potential, possibly through its ability to protect liver and renal architectures (Shah *et al.* 2020).

Rauwolfia tetraphylla is an Ayurvedic and Unani medicinal plant with potential applications for treating various ailments. Singh *et al.* evaluated the anticonvulsant activity of ethanolic extract of *R. tetraphylla* leaves. The maximal electroshock seizure (MES) model and pentylenetetrazole (PTZ)-induced seizure model were used to screen the anticonvulsant activity in Swiss albino mice. The extract was evaluated for rutin and gallic acid content using HPTLC. It revealed that the extract contained 15.60% rutin and 7.81% gallic acid. The extract (100–800 mg/kg) significantly reduced the duration of MES-induced seizures and

protected the animals from PTZ-induced tonic seizures. It indicated that *R. tetraphylla* leaves possess significant anticonvulsant activity, highlighting the potential of this plant for the treatment of seizures (Singh *et al.* 2019).

Macrofilaricidal activity was analysed by four different leaf extracts such as hexane, chloroform, acetone, and methanol of *R. tetraphylla* against bovine filarial parasite *Setaria cervi*. It is assessed by worm motility assay, MTT reduction and Glutathione-S-transferase (GST) enzyme inhibition assay against female worms of *S. cervi*. Worm motility readings take place at 1hr interval up to 24 h after treatment with extracts and standard (Diethylcarbamazine- DEC). Methanol extracts showed significant activity among all the extract of reduction 89.28% at 10 mg/mL. DEC showed relatively less active than methanolic extract with 79.22% activity in the similar dosage. The IC₅₀ value for methanolic extract was set up to 0.03 mg/mL, which was enhanced than standard (2.84 mg/mL). The GST enzyme activity of *Setaria* worms was inhibited by methanolic extract by 56.41% at 10 mg/mL as compared to untreated worms. Methanolic extract drastically killed the oocytes of *Setaria* worms as all the oocytes stained blue in treated worms. Methanol extract showed maximum reduction value in MTT, GST level and enhanced activity in comparison to the standard drug in terms of all the assays (Behera and Bhatnagar, 2017).

Platelet antiaggregants (PAAs) are useful for the prevention of thrombus formation especially for coronary thrombosis, cerebral thrombosis, venous thrombi, and haemodialysis. Traditionally used 17 plants in Guatemala were screened for the treatment of blood disorder and infectious diseases, for platelet antiaggregant activity. Aqueous extract of 17 plants were used for assay. Aggregatory method was measured by turbidimetric method of Born and Cross, in which 17 extracts were tested in that eight extracts had a pronounced inhibitory effect on the aggregation of washed human platelets induced by 0.075 U/mL of thrombin was inhibited by extracts of *R. tetraphylla*, *Tagetes lucida*, *Tecoma stans*, *Bixa orellana*, *Tridax procumbens*, *Annona reticulata*, *Petiveria alliacea* and *Neurolaena lobata*. The remaining extracts did not have apparent inhibitory effects, and certainly caused some platelet aggregation themselves, in the lack of thrombin (Villar *et al.* 1997).

2.17. Biotechnological Applications and Nanotechnology

Hemashekhar *et al.*, (2019) evaluated the synthesis of gold nanoparticles (AuNPs) using an endophytic fungus *Alternaria* spp. extract derived from the *R. tetraphylla* root. The synthesized AuNPs were characterized using various techniques such as UV, Energy Dispersive x-ray Spectroscopy (EDX), and TEM to confirm their variable shapes and an average particle size of 28 nm. The characterized AuNPs were evaluated for antibacterial

activity against *E. coli*, *P. aeruginosa*, *K. pneumonia*, and *S. aureus*. The AuNPs were also tested for their antioxidant, antibacterial, and antimetabolic activities. It showed that the synthesized AuNPs exhibited significant antibacterial, antioxidant, and antimetabolic activities. It provided a novel method for the synthesis of AuNPs using the *R. tetraphylla* based endophytic extract as a reducing agent and suggested that the synthesized AuNPs may have potential applications in various fields, including medicine and biotechnology (Hemashekhar *et al.* 2019).

Vinay *et al.* (2019) developed a novel, eco-friendly method to synthesize silver nanoparticles (Ag NPs) using *R. tetraphylla* leaves extract as a green reducing and stabilizing agent. The synthesized Ag NPs were characterized using various analytical techniques, revealing a cubic phased silver nanoparticle with an average size of 40 nm and a spherical shape. The antioxidant activity of the material was examined, showing an inhibitory concentration of 82.13 µg/mL against DPPH free radical. Moreover, the cytogenetic effect of the Ag NPs was tested on the root cells of *Allium cepa*, indicating antimetabolic activity and precise chromosomal aberrations such as chromosome-breaks, chromosome-stickiness, laggard chromosome, clumped chromosome. Due to the discharge of Ag⁺ ions and production of Reactive Oxygen Species (ROS), the Ag NPs showed great anticancer activity. It provided an effective and eco-friendly method for synthesizing Ag NPs, which have potential applications in various fields (Vinay *et al.* 2019).

The synthesis of Ag NPs from *R. tetraphylla* leaves extract using a green approach technique. The synthesized nanoparticles were characterized using various analytical techniques including X-ray diffraction, FTIR, UV, SEM, EXD, and TEM analysis. The nanoparticles were found to be in the cubic phase with an average particle size of about 40 nm and a spherical shape. The material was further examined for photocatalytic degradation of environmental pollutants such as Methylene blue (M.B), Rhodamine B (Rh. B), and Rose bengal (R.B), with degradation efficiencies of 81%, 55%, and 80%, respectively. The nanoparticles demonstrated superior anti-bacterial activity against gram-positive bacteria like *P. aeruginosa*, *E. coli* and *Klebsiella aerogenes*, and gram-negative bacteria *S. aureus*. The nanoparticles showed blue emission upon excitation at 370 to 400 nm, which makes it useful for blue LED (light-emitting diode) applications. It presents the synthesis of Ag NPs using *R. tetraphylla* leaf extract as a natural reducing agent for Luminescence and Dye degradation applications, making it an effective and eco-friendly alternative to traditional methods (Vinay *et al.* 2019).

Rauvolfia tetraphylla leaf extracts have been explored for the biological synthesis of Ag NPs. The synthesized Ag NPs were analysed using various spectroscopic and imaging techniques, including UV, SEM, XRD, EDX, and FT IR. It showed that the bioreduction of silver ions by *R. tetraphylla* leaf extract resulted in the synthesis of nanoparticles with spherical, triangle, and square shapes, ranging in size from 26 – 37 nm. FT-IR spectrum confirmed the involvement of various organic compounds, such as aromatic amines, amide (I) and amide (II) groups, flavonoids, polyols, and secondary alcohols, in capping and reduction of silver nanoparticles. Phytosynthesized silver nanoparticles exhibited antibacterial activity against *S. aureus*, *E. coli*, and *P. aeruginosa*. The biosynthesis of Ag NPs using leaf extract appears to be a cost-effective, eco-friendly, and easy alternative to other conventional methods for nanoparticle synthesis (Kalaiarasi *et al.* 2013).

2.18. Endophytic Fungi

Endophytic fungi are microorganisms that reside within the tissues of plants and can produce a range of secondary metabolites like those found in their host plant. Different parts of *R. tetraphylla* were examined for their endophytic fungal content. The isolated endophytic fungi were identified morphologically and their isolation rates, colonization rates, and relative frequencies were calculated. A total of 26 endophytic fungi were isolated and identified, including species from the genera *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Cylindrocephalum*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Pestalotiopsis*, *Thielavia terricola*, and *Mycelia sterilia*. Two of the identified fungi, *Curvularia* sp. and *Aspergillus* sp., were selected and grown in rice medium to produce secondary metabolites. These extracts were screened for antimicrobial activity against *S. aureus*, *B. cereus*, *P. aeruginosa*, *E. coli*, *S. typhimurium*, and *Candida albicans*. The extract of *Curvularia* sp. showed effective inhibition against all tested organisms, while the extract of *Aspergillus* sp. was effective against all except *P. aeruginosa*. Phytochemical analysis of the extracts revealed the presence of alkaloids, terpenoids, and tannin compounds. It revealed the diverse range of endophytic fungi associated with *R. tetraphylla* and their potential as sources of antimicrobial secondary metabolites (Alurappa & Chowdappa, 2018).

2.19. Biosynthesis of Alkaloids

The biosynthesis of MIAs in the genus *Rauvolfia* involves complex biochemical pathways. Despite extensive research on the subject, many reaction steps involving oxygenases and oxidoreductases remain intangible. Geissler *et al.* reported on the molecular

cloning and characterization of three cinnamyl alcohol dehydrogenase (CAD)-like reductases from *R. serpentina* cell culture and *R. tetraphylla* roots. Functional analyses of the recombinant proteins with a range of MIAs as potential substrates, which led to the identification of one enzyme as a CAD, which is likely involved in lignin formation. The remaining two reductases were found to be isoenzymes derived from orthologous genes of the *Rauvolfia* species. Their catalytic activity involved the specific conversion of vomilenine to 19, 20-dihydrovomilenine. It suggested the existence of a previously unknown bypass in the biosynthetic pathway to ajmaline, a structurally diverse MIA (Geissler *et al.* 2016).

The biosynthesis of ajmalicine, an important indole alkaloid in *R. verticillata*, involves several enzymatic steps. TDC is a crucial enzyme that converts tryptophan into tryptamine, the indole moiety of ajmalicine. The full-length cDNA of *RvTDC* was characterized and found to encode a 499-amino-acid polypeptide. Recombinant *RvTDC* was able to convert tryptophan into tryptamine, with a K_m value of 2.89 mM, indicating lower affinity than other plant TDCs. This suggested that TDC may be the committed-step enzyme involved in ajmalicine biosynthesis in *R. verticillata*. The expression of *RvTDC* was found to be highest in hairy roots, with a 109-fold higher expression level than in roots. MeJA (Methyl jasmonate) treatment slightly upregulated the expression of *RvTDC* and resulted in increased ajmalicine production in hairy roots. The expression levels of the five MEP (2-C-methyl-D-erythritol 4-phosphate) pathway genes, strictosidine synthase and strictosidine glucosidase did not respond positively to MeJA, while STR (Strictosidine synthase) was sharply downregulated. The highest content of ajmalicine was found in hairy roots, followed by bark and young leaves. The expression level of *RvTDC* was positively correlated with the accumulation of ajmalicine, indicating that TDC is a key enzyme involved in ajmalicine biosynthesis in *Rauvolfia*. It sheds light on the biosynthesis pathway of ajmalicine and provided the regulation of TDC expression and ajmalicine production of *Rauvolfia* (Liu *et al.* 2012).

Rauvolfia serpentina is a plant species known for its bioactive MIAs and is endangered due to overexploitation. The commercial and economic importance of this plant is largely attributed to its MIAs. To address the need for a validated and rapid method for quantifying these MIAs, UHPLC-QqQLIT-MS/MS method in the multiple reaction monitoring (MRM) mode was analysed. It determined bioactive MIAs in ethanolic extracts of seven *Rauvolfia* species and herbal formulations. The validated method demonstrated good linearity, precision, stability, and recovery. It was applied to quantitate MIAs and showed that *Rauvolfia vomitoria* had a high content of ajmaline and serpentine, while *R. tetraphylla* had

high quantities of yohimbine and ajmalicine. Reserpine was detected in high content in *R. micrantha* and *R. serpentina*. It provided an important tool for selecting suitable *Rauvolfia* species based on the abundance of MIAs (Kumar *et al.* 2016).

The identification and characterization of MIAs in the roots of *Rauvolfia* species is of great interest due to the medicinal importance of these compounds. Two MIAs, yohimbine and reserpine, are included in the Model List of Essential Drugs of the WHO due to their aphrodisiac, antihypertensive, and tranquilizing properties. UHPLC coupled with Orbitrap Velos Pro hybrid mass spectrometry was used to identify and characterized MIAs in crude extracts of six *Rauvolfia* species. HRMS/MS spectra of the standard compounds yohimbine and reserpine were evaluated to identify the MIAs in the extracts. Two modes of fragmentation, higher energy collisional dissociation (HCD) and collision-induced dissociation (CID), were analysed to produce diagnostic fragment ions. CID-MS/MS mode provided significantly more structural information about the basic skeleton of the MIAs, making it the recommended mode for analysis. Six diagnostic fragmentation pathways were established by multi-stage mass analysis to provide information about the substitution of the MIAs. The proposed diagnostic fragmentation pathways were successfully applied to identify and characterize ten bioactive reserpine classes of MIAs in the crude root extracts of the six *Rauvolfia* species. It provided the identification and characterization of MIAs in *Rauvolfia* species using HRMS/MS spectra and CID-MS/MS mode of fragmentation (Kumar *et al.* 2016).

2.20. Molecular Taxonomy

Molecular analysis in different accessions of *R. tetraphylla* were reported from seven different locations in Andhra Pradesh, India. The plant was identified and collected based on the flora of A.P. Molecular analysis was evaluated by RAPD analysis and it involved two steps such as PCR amplification and agarose gel electrophoresis. Forty primers of arbitrary sequence provided by Operon Technology were tested for PCR amplification. RAPD data generated with forty primers was accumulated as a binary matrix for cluster analysis using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System). Analysis of seven accessions of *R. tetraphylla* with a total of forty primers revealed 51.6% of polymorphism. The number of scorable polymorphic markers generated was 205 out of 397 total markers. Unique marker was found at 51.6% of polymorphism. The genetic distance ranges from 0.816 to 0.932 based on the levels of genetic diversity within accessions. It showed that high

levels of differentiation among accessions exists independent of geographical distance in cross-pollinated plants (Padmalatha, 2006).

2.21. Genomic Studies Transcriptomics

The genetic diversity of *R. serpentina* with 19 accessions were collected from different regions of India and analysed using random amplified polymorphic DNA (RAPD). Out of 60 RAPD primers tested, 15 (25%) were the most informative and produced multiple band profiles with 7 to 11 amplified DNA fragments. Of the 143 amplified bands, 95 (66.46%) were polymorphic, with an average of 6.33 polymorphic fragments per primer. Jaccard's genetic similarity coefficient ranged from 0.313 to 0.875. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram showed two main clusters that split at a Jaccard's similarity coefficient of 0.313. It could be useful in developing conservation strategies for this valuable medicinal plant (Subramanyam *et al.* 2013).

Zheng *et al.* described the cloning and characterization of two genes, MECS (2-C-Methyl-D-erythritol-2,4-cyclodiphosphate synthase) and HDS (1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase), involved in MEP pathway of *R. verticillata*. The full-length cDNA sequences of *RvMECS* and *RvHDS* were obtained and submitted to Gene Bank. Bioinformatic analysis revealed that both genes had homology with MECSs and HDSs from other plant species and contained a conserved transit peptide for plastids. Phylogenetic analysis indicated that *RvMECS* and *RvHDS* belonged to plant MECSs family and HDSs family, respectively. Quantitative PCR showed that *RvMECS* and *RvHDS* had different expression patterns across different tissues, with *RvMECS* expression being highest in flowers, and *RvHDS* expression being highest in flowers and leaves. Furthermore, expression of *RvMECS* was up-regulated by exogenous elicitors, while *RvHDS* expression was inhibited by elicitors. It provided valuable insights into the molecular genetics of the MEP pathway in *R. verticillate* (Zheng *et al.* 2011).

The elucidation of MIAs biosynthesis has been developing in Apocynaceae family through transcriptomic analyses and reverse genetic approaches such as virus-induced gene silencing (VIGS). While these tools have been primarily adapted for *C. roseus*, the VIGS procedure has been underutilized in other Apocynaceae species, including *Rauwolfia* sp. *Rauwolfia* species are unique sources of valuable MIAs, such as reserpine, and are well-known models for studying alkaloid metabolism. Therefore, an efficient VIGS procedure would be beneficial for studying these species. Recent modifications in the inoculation method of Tobacco rattle virus vectors via particle bombardment have allowed for the

biolistic-mediated VIGS approach to be readily used in both *R. tetraphylla* and *R. serpentina*. By establishing bombardment conditions that minimize injuries to transformed plantlets, gene downregulation efficiency was evaluated at approximately 70% expression decrease in both species by silencing the phytoene desaturase encoding gene. This gene silencing approach will be crucial in identifying and characterizing genes involved in alkaloid biosynthesis in *Rauvolfia* species (Corbin *et al.* 2017).

Genetic variability of *R. tetraphylla* collected from five different locations in Tirunelveli hills in Tamil Nadu has been carried out using RAPD. DNA has been isolated and then RAPD analysis has been carried out. The quality and its amount were assessed by UV spectrophotometer. Eight primers were tested, in which five primers were selected which produce reproducible bands. Agarose gel electrophoresis was used to separate the amplification products. It is visualized by UV transilluminator and photographed with gel documentation system Alpha Imager 1200. Five primers were screened, out of which 27 scorable polymorphic markers were generated. The genetic distance among the population ranged from 0.0770 to 0.3514 and the genetic identity ranged from 0.7037 to 0.9259. Thus, the observed and valuable number of alleles is about 0.5007 and 0.3690, respectively. It is obvious that there is discrete genetic variability in *R. tetraphylla* and is required on the implications of reduced genetic variation for survival and fertility (Mahesh *et al.* 2008).

Rauvolfia serpentina were evaluate the potential of molecular identification by searching for species-specific DNA polymorphisms, which resulted in the discovery of two species-specific indels in the rps16 intron region for *R. serpentina*. The DNA barcoding method was then tested for its specificity, reproducibility, sensitivity, and stability using samples of various tissues and ages that had been treated differently for preservation. The tested DNA extractions in a range of amplification settings and dilutions. Species-specific rps16 intron sequences were obtained from 79 herbarium accessions and one confiscated root, encompassing 39 different species. It highlights the potential of molecular analysis for the forensic identification of Indian snakeroot, as demonstrated by the obtained species-specific rps16 intron sequences from various tissue and age samples of 39 different species (Eurlings *et al.* 2013).