Assessment of Genetic and Phytochemical Variations of *Leptadenia reticulata* from Different Geographical Regions of India

Thesis submitted to

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH (KAHER), BELAGAVI

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For the award of the degree of

DOCTOR OF PHILOSOPHY

In the Faculty of

Inter-disciplinary Science

By

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(Registration No: KAHER/Ph.D./20-21/DO1220062)

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2023

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Mr. Roshan Kumar Sharma

LIST OF ABBRAVATIONS

BLAST : Basic Local Alignment Search Tool

AMOVA : Analysis of Molecular Variance

Bar-HRM : Barcoding - High Resolution Melting

bp : Base pair

CTAB : Cetyltrimethylammonium bromide

CV : Coefficient of variation

DMSO : Dimethyl sulfoxide

EDTA : Ethylene diamine tetra acetic acid

EMR : Effective Multiplex Ratio

EtBr : Ethidium bromide

GC-MS : Gas chromatography–mass spectrometry

G_{ST} : Genetic differentiation

h : Nei’s genetic diversity

HPLC : High-performance liquid chromatography

HP-TLC : High-performance thin-layer chromatography

HRM : High Resolution Melting

H_S : Heterozygosity within population

H_T : Total heterozygosity

I : Shannon's Information Index

ICH : International Council for Harmonisation

ICUN : International Union for Conservation of Nature

IDT : Integrated DNA Technologies

ISSR : Inter Simple Sequence Repeat

ITS2 : Internal Transcribed Spacer 2

LOD : Limit of detection

LOQ : Limit of quantification

MEGA : Molecular Evolutionary Genetics Analysis

MI : Marker Index

***N*a** : Observed number of alleles

NCBI : National Center for Biotechnology Information

***N*e** : Effective number of alleles

***N*m** : Gene flow

PB : Number of polymorphic bands

PCA : Principal Component Analysis

PCoA : Principal Coordinate Analysis

PCR : Polymerase Chain Reaction

PEG : Polyethylene glycol

PIC : Polymorphism Information Content

Pop : Population

PVP : Polyvinylpyrrolidone

R^2 : Correlation coefficient

RAPD : Random Amplified Polymorphic DNA

***rbc*L** : Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

R_f : Retention factor

RSD : Relative standard deviation

RT-PCR : Real-Time Polymerase Chain Reaction

SCAR : Sequence Characterized Amplified Region

SSR : Simple Sequence Repeat

TAE : Tris-acetate-EDTA

Tm : Melting temperature

UBC : University of British Colombia

UPGMA : Unweighted Pair Group Method with Arithmetic average

WHO : World Health Organization

WII : Wildlife Institute of India

// ABSTRACT

ABSTRACT

Background:

Leptadenia reticulata (Retz.) Wight & Arn, a highly valued and extensively traded medicinal plant, plays a crucial role in Ayurvedic practices, primarily for its revitalizing, rejuvenating, and lactogenic properties. However, because of the established medicinal properties and increase in demand across the world, the plants are being overexploited and are rapidly decreasing in number. It is therefore important to carry out population genetic studies on the plant to understand the existing variations in their gene pool and their correlation with respective phytoconstituents so that appropriate conservation strategies can be planned. Depleting numbers, and inadvertent mistakes in identification coupled with rampant substitution and adulteration has necessitated the development of better tools for correct identification and differentiation of this medicinally important plant from common adulterants/substitutes.

Objectives: (i) To study the DNA profile of *Leptadenia reticulata,* from different geographical regions of India using ISSR/RAPD primers. (ii) To investigate the phytoconstituent variation in *Leptadenia reticulata* using the fingerprinting method. (iii) To develop the molecular markers for identification and differentiation of *Leptadenia reticulata* from its adulterants and substituents.

Methodology: Fifty-five accessions of *L. reticulata* were collected from various geographical regions of India. Furthermore, common adulterants of *L. reticulata* including *Wattakaka Volubilis* and *Holostemma ada-kodien* were gathered from various locations. Genetic diversity markers including Inter Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) were employed to determine the genetic variations while High-performance thin-layer chromatography (HP-TLC)

based quantification for quercetin was used in the study. DNA barcoding was used to differentiate the *L. reticulata* from its adulterants/substituents. Further, internal transcribed spacer 2 (ITS2) based primers were developed to detect and differentiate adulterants/substituents of *L. reticulata*.

Results: Both the marker systems (RAPD and ISSR) showed 100% polymorphism. The genetic assessment showed that diversity was relatively high at the species level but lower at the population level. Analysis of Molecular Variance (AMOVA) indicated genetic variation within *L. reticulata* populations (89% for ISSR and 97% for RAPD) and comparatively less variation among populations (11% for ISSR and 3% for RAPD). Limited genetic differentiation and moderate gene flow were observed between the populations of *L. reticulata*. Two separate groups have been generated by the Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) genotype clustering approach. A simple, sensitive, and accurate HPTLC method was developed and validated for quantifying quercetin in *L. reticulata*. Using the HPTLC method, quercetin content variation was observed in *L. reticulata* samples. Further, ITS2-derived developed markers were effective in distinctly identifying *L. reticulata* from common adulterants such as *W. volubilis* and *H. ada-kodien*.

Conclusion: Genetic diversity observed within *L. reticulata* populations, the moderate gene flow and high interspecies diversity are promising for advocating the selection of varied genotypes from multiple locations for both ex-situ and in-situ conservation programs. As per HPTLC observation, geographic origin and climatic conditions may be major factors in determining the compound variation in *L. reticulata* accessions. The DNA barcoding method proved to be an effective tool for differentiating this species from its adulterants. The ITS2-derived DNA barcoding combination with High-Resolution Melting (Bar-HRM) analysis, conducted on this species, demonstrated

sufficient sensitivity to detect and differentiate *L. reticulata* from common adulterants like *W. volubilis* and *H. ada-kodien*, suggesting its potential application in determining drug content in crude samples.

Keywords: Genetic diversity; *Leptadenia reticulata*; Conservation; DNA fingerprinting; ISSR; RAPD; DNA barcoding; HRM analysis; Adulteration, HPTLC

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Chapter-1
Introduction

1. Introduction

1.1. Background

Leptadenia reticulata (Retz.) Wight & Arn., a prominent herb in Ayurvedic medicine, belongs to the Apocynaceae family.[1] In Ayurveda, *L. reticulata* herb is called by different names, such as Jivanti, Jivaniya, Jivapushpa, Hemavati, Jivana, Shakashreshtha, Payaswini, Maangalya, and Madhusrava. It is also known as Keerippaalai in Siddha.[2] It is referred to by several regional names in different parts of India, such as Jiwanti or Jeevanti in English, Dori in Hindi, Bhadjivai in Bengali, Methidodi, Dodi saka/Dodi Saag, Dori in Gujarati, Haranvel, Hiranvel in Marathi, Hiriyahalle in Kannada and Palaikkodi in Tamil.[2] Ayurveda's Rasayana science, which focuses on enhancing overall health, vigur, and vitality, recognizes *L. reticulata* (Jivanti) for its influential properties including revitalizing, rejuvenating, and lactogenic.[3] Classical Ayurvedic texts acclaim Jivanti as a crucial vegetable (ṣreṣṭha śāka) for maintaining a healthy state.[4]

Traditionally, this plant has been used for treating a variety of health conditions. This climber's stem, often used in decoctions, aids in the expulsion of the placenta post-birth and helps to control bleeding post-circumcision.[5] The roots are beneficial for treating coughs, asthma, tuberculosis, and various skin infections, as well as for overall weakness.[6] The flowers are known to improve eyesight[7], while the seeds, often used in paste form, are applied to gangrenous areas.[5] The entire plant serves multiple purposes, including as an antiabortifacient, tonic, restorative, bactericide, antipyretic, wound healer for prostitutes, and as a remedy for mouth ulcers.[8] *L. reticulata* is also effective against the 'tridoshas' (Vatta, Pitta, and Kapha) in Ayurvedic medicine, aiding in the treatment of general debility, involuntary seminal discharge, and snake bites.[9, 10] It is

also used to alleviate symptoms of hematopoiesis, dysentery, emaciation, dyspnea, burning sensations, and night blindness.[3] The plant contains a variety of chemicals like phenolic, glycosides, and flavonoids, according to a phytochemical examination. *L. reticulata* has been shown to contain important bioactive chemicals that have been extracted and shown to have substantial pharmacological activity. These components include α-amyrin, β-amyrin, ferulic acid, diosmetin, rutin, quercetin, β-sitosterol, stigmasterol, hentriacontanol, simiarenol, apigenin, alkaloids, and phenolic compounds. Furthermore, flavonoids have been utilized to lower the probability of heart disease and cancer.[12, 13] Quercetin, one of these compounds, is known for treating various ailments including cancer, cardiovascular diseases, allergies, and diabetes, and has immune-suppressive and anti-infective activities.[14, 15]

The Ayurvedic herb *L. reticulata* is a member of the Apocynaceae plant family. Its taxonomic position is detailed (Kingdom: Viridiplantae, Phylum: Streptophyta, Class: Gentianales, Order: Magnoliopsida, Family: Apocynaceae, Genus: *Leptadenia,* Species: *reticulata*). The *L. reticulata* species is characterized as a robust, woody climber. Its foliage presents as opposite, heart-shaped to ovate, spanning 4 to 7.5 cm in length and 2 to 5 cm across. The leaf margins are smooth, ranging from sharply pointed to slightly pointed or blunt with a tiny projection, and the base is evenly shaped; notably, stipules are not present. The leaf stalk, or petiole, measures between 0.8 and 2.5 cm in length. The leaf blade itself is broadly ovate to elongated ovate, with dimensions of 3-9 cm by 1.1 cm, featuring a base that is either rounded or blunt and a tip that is pointed or slightly elongated. As the leaf matures, it becomes nearly smooth. The plant's stem is tubular, sometimes curved, and extensively branched, with newer branches being smooth. The bark exhibits a cork-like texture, is rough to the touch, displays

longitudinal ridges and wrinkles, has deep fissures, and is a yellowish-brown hue. The flowers of *L. reticulata* are hermaphroditic, symmetrical, composed of five segments, and star-shaped, measuring 6-8 mm across. They are supported by a short stalk. The calyx segments are ovate, measuring 1-1.7 mm in length, and covered in dense, short hairs. The corolla features a tube approximately 3-3.5 mm in length, with short, ovate-oblong lobes that are longer than the tube, ridged, and have an inward-facing tooth. These are greenish or orange-yellow, densely woolly, and covered in short hairs on both sides. The corona lobes are small and fleshy. The anther curves over the stigmatic head, creating a short gynostegium. The ovary of the plant is positioned above the other flower parts. The fruit of the *L. reticulata* consists of a pair of slender, egg-shaped to elongated, semi-woody, and swollen follicles, each measuring 6-8 cm by 2-2.5 cm, with a tapering to blunt tip.

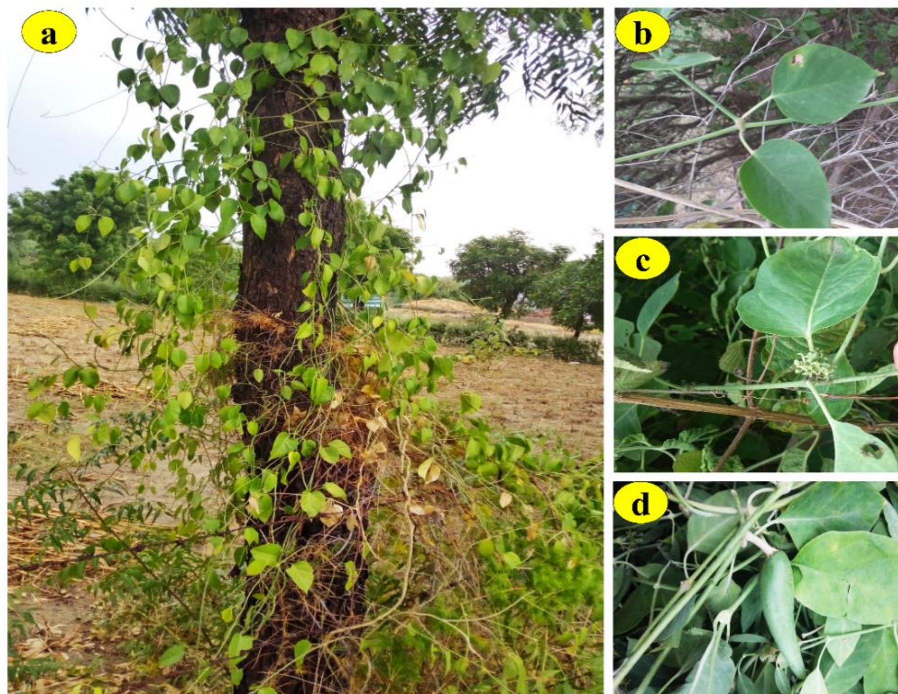


Figure 1: Habitat of *Leptadenia reticulata* (Retz.) Wight & Arn (a: Whole climber; b: Leaf; c: Flower; d: Fruit).

These fruits are smooth and contain numerous seeds. The seeds themselves are egg-shaped and flattened, equipped with a tuft of long, silky hairs at one end, aiding in their dispersal. *L. reticulata* typically grows rapidly from April to September. Its flowering season is from May to July, and fruits from November to February. Seed maturation occurs from December to May, and seed germination is observed from June to September.[17, 18] The habit of *L. reticulata* (Retz.) Wight & Arn. is depicted in Figure 1.

In the genus *Leptadenia*, there are four species, including *Leptadenia reticulata* (Wight & Arn.), *Leptadenia pyrotechnica* (Forssk.), *Leptadenia arborea* (Forssk.), and *Leptadenia hastata* (Pers.).[19] The origin of *Leptadenia reticulata* is shrouded in some mystery, but its initial documentation in the ancient Atharva Veda hints at an Indian origin. Mainly, *L. reticulata* is available in tropical and subtropical areas of Asia and Africa, including countries like Mauritius, the Malayan Peninsula, the Philippines, Sri Lanka, the Himalayan region, Burma, Myanmar, Nepal, Cambodia and Madagascar.[5, 16, 17]

In the Indian subcontinent, the distribution of *L. reticulata* is quite extensive. It is found in various regions such as Rajasthan, Gujarat, the Khasia Hills, the Nilgiris, Sikkim, Tamil Nadu, Karnataka, and the sub-Himalayan tracts of Punjab and Uttar Pradesh. Its presence is also noted across the Deccan Peninsula, with altitudes reaching up to 900 meters. The species extends to Andhra Pradesh, Bihar, Delhi, Goa, Haryana, Kerala, Madhya Pradesh, Maharashtra, Orissa, and the Laccadive Islands.[16, 17] In Rajasthan, it is particularly observed in areas like Manai (Jodhpur), Kaccholi (Sirohi), Jhalawar, around Jaisamand Lake, and in the Aravali range. There is a notable cultivation of this plant near Jodhpur, Rajasthan, primarily due to its significant medicinal demand.[1]

L. reticulata is typically found in dry secondary forests, dry savannas, or sandy coastal regions, ranging from sea level to an elevation of 2700 meters.[5] It thrives in climates that experience a mix of rainy seasons and prolonged hot, dry periods. The plant is adaptable to various soil types, including sandy loam, alluvial black soils, and red laterite soils. It prefers mild weather conditions and requires abundant sunlight for optimal growth.[20]

The practice of using plants for medicinal purposes is as ancient as humanity itself, forming an integral part of various traditional medicine systems such as Ayurveda, Homeopathy, Siddha, Unani, Naturopathy, traditional Chinese, Tibetan, and Native American medicine.[21-24] Almost 80% of the population globally acquires their primary medical care through herbal remedies, as reported by the World Health Organization (WHO).[21, 23, 25] Right now, natural plant resources and their components are the primary thrust of drug development studies. The majority of therapeutically efficacious medications on the market today were found using information gleaned from numerous conventional methods of treating illness.[22-25, 26]

In India, the demand for medicinal plants like *L. reticulata* has surged both domestically and internationally. *L. reticulata*'s dry powder costs INR 211/kg, while its blossoms are valued at INR 80/kg.[1, 27] *L. reticulata* powder, which is 100% natural, typically costs about $8 on the market.[28-30] In 2006–2007, the dry roots were marketed at INR 65/kg, with a 23 tons/year demand from the market.[29, 30] Various formulations and products containing *L. reticulata* are available in the market *viz.*, Confido, Speman, GalactinVet, Spemanfortevet, Speman vet, Himalaya™ Chyavanprasha (company: Himalaya Drug House), Calshakti (company: Intas), Safe herbs (company: VASU Healthcare), Jivanti Powder/capsule (company: Evaidyaji Wellness), Enviro Care (company: Satveda,

India/Herbs Forever), Antisept (company: Zydus Cadila) and Praas™ (Chyawanprash) (company: Komal Herbals).[2] The immense medicinal value and overexploitation of *L. reticulata* have led to its fragmentation in India, where it once grew abundantly. It is now classified as 'vulnerable' by the International Union for Conservation of Nature (IUCN).[31] The limited wild populations are insufficient to meet the growing commercial demand, emphasizing the need for conservation to prevent potential extinction. Essential elements of conservation initiatives include the preservation and long-term utilization of plant species. To effectively establish conservation measures, it is essential to comprehend the genetic diversity both within and among populations of rare or vulnerable plant species.[32, 33] The loss of genetic diversity may seriously threaten the survival of these species. Gaining insights into the genetic structure and changes within a species is fundamental for crafting conservation and resource utilization plans.[34] Recognizing the patterns of variation among individual plants and across populations is essential for formulating genetic management strategies.[35, 36] Genetic changes, influenced by mutation, drift, selection, gene flow, and other factors, drive the process of biological evolution.[37, 38] For assessing genetic variations, a range of genetic markers are available, each with its advantages and limitations. Techniques such as RAPD and ISSR are popular due to their simplicity, cost-effectiveness, and sensitivity.[32, 39-41] These methods are widely used in genetic studies. For chemical constituent analysis, techniques like HPTLC and HPLC have been recognized as efficient, accurate, and cost-effective, establishing themselves as standard methods for plant constituent estimation.[42-44]

The use of indigenous plants in traditional medicine is significant, but the sector faces challenges due to increasing incidents of adulteration, often driven by high demand or

misidentification, and sometimes even intentional. Misidentification can lead to unintentional substitution, while intentional adulteration typically involves replacing a more expensive herb with a cheaper one. *L. reticulata*, widely used in traditional medicine, is an example of a plant that is rapidly becoming endangered due to overharvesting and the risk of ineffective products due to the absence of authentic ingredients. The plant is often adulterated or substituted with other herbs like *W. volubilis*, *H. ada-kodien*, and *Dendrobium macraei*.[4]

For quality control, pharmacognostic assays are commonly used, but these methods are labour-intensive and time-consuming. Therefore, DNA-based technologies, that are a more stable and reliable, have clear advantage over other strategies. Alongside molecular authentication, chemical profiling has become essential for species identification and quality control.[45, 46] However, challenges arise due to the similarity in morphology and non-specific chemical constituents of botanicals, which can lead to misidentification. Additionally, DNA degradation in processed products can complicate DNA-based assays. Therefore, a combined approach of genetic and chemical identification is ideal for accurate identification and authentication.[45]

1.2. Review of literature

L. reticulata, a prominent herb in Ayurvedic medicine, is renowned for its health-sustaining properties. Jivanti, as it is most commonly known, has been described as "one which maintains a healthy state. Charaka mentioned it as the best wholesome vegetable (Shreshtha Shaka) to be consumed for maintaining good health.[48] Ayurvedic literature, dating from the Veda (6000 BC) to Samhita (1500 BC – 600 AD), and later texts like Nighantu and Sangraha granthas (800AD – 1900AD), have documented the use of

herbal remedies, including Jivanti.[49] Pharmacologically, Jivanti is classified into various categories such as Jeevaniya gana (rejuvenating herbs), Vayasthapana gana (anti-ageing drugs), Madhura skandha (sweet property drugs), Shaka varga (vegetables), Kakolyadi gana, and Jeevaniya panchamula in classical texts. In Ayurveda, it is utilized as a standalone herb for its Jivaniya (longevity promoting), Snehopaga (oleating), Shvasahara (anti-asthmatic), Vayasthapana (anti-aging), Rasayana (rejuvenating), and Chakshushya (eye tonic) properties. *L. reticulata* also forms a part of numerous polyherbal or herbomineral formulations.[50]

1.2.1. Synonyms of Jivanti[50]

According to karma, Jivanti is referred to by several names and purposes. Included in them are Jeevanti, which preserves health, Jeevani, Jeeva, and Jeevada, which provide long-lasting Rasayana effects, Jeevanneya, a good tonic, Jeevavrdhini, which bestows health and vitality, and Bala Vardhini, which provides strength.

1.2.2. Taxonomical nomenclature of *L. reticulata* [2]

Plant profile	
Kingdom	Viridiplantae
Phylum	Streptophyta
Class	Gentianales
Order	Magnoliopsida
Family	Apocynaceae
Sub-family	Asclepiadaceae
Genus	*Leptadenia*
Species	*reticulata*

1.2.3. Distribution and habitat[2, 19]

Although the true origin of *L. reticulata* has yet to be identified, its description in the Atharvaveda, the oldest scripture of Hinduism, suggests that it likely originated in India. This plant is widely distributed across various Indian regions, including Rajasthan, Gujarat, Punjab, along the Himalayas, in the Khasi Hills, Sikkim, the Deccan Plateau, the Konkan belt, as well as in Karnataka and Kerala, thriving at elevations as high as 2000 meters. Its range of distribution is not limited to the Indian subcontinent; it also includes tropical and subtropical regions in Africa, the Malay Peninsula, Myanmar, Nepal, Sri Lanka, Cambodia, the Philippines, Mauritius, and Madagascar. Particularly in Gujarat and the Kathiawar region, *L. reticulata* is popularly cultivated as a kitchen herb. After intensive field survey in 12 districts in Western Rajasthan (Indian Thar Desert), Panwar and Tarafdar reported the occurrence of *L. reticulata* from various districts. This species was also found on hedges, in open forests, and on the lower slopes of hills.

1.2.4. Morphology[2, 19]

L. reticulata, scientifically recognized as a robust woody climber, displays distinctive botanical features. Its leaves exhibit an opposite arrangement, and their shapes vary from ovate to cordate, measuring approximately 4 to 7.5 cm in length and 2 to 5 cm in width. Notably, these leaves possess a symmetrical base and are devoid of stipules. The petiole, which serves as the leaf's stalk, typically ranges from 0.8 to 2.5 cm in length. The leaf blade, broadly ovate to ovate-oblong, exhibits dimensions of 3 to 9 cm in length and 1.1 cm in width. It possesses a rounded to obtuse base and can terminate in an apex that is either acute or short acuminate. The stem of *L. reticulata* is characterized by its

cylindrical shape, occasionally displaying a bent configuration. It extensively branches out, with the younger branches being smooth and lacking hair. The bark of this plant species is reminiscent of cork, featuring a rough texture, longitudinal ridges, wrinkles, and deep cracks. It takes on a distinctive yellowish-brown hue. Moving on to its reproductive characteristics, the flower of *L. reticulata* is bisexual, exhibiting a regular, 5-merous structure. These star-shaped flowers typically measure between 6 to 8 mm in diameter and are supported by a short pedicel. The calyx lobes of the flower are ovate and measure approximately 1 to 1.7 mm in length, displaying a dense covering of short hairs. The corolla, on the other hand, presents a 3 to 3.5 mm long tube, accompanied by a short lobe that is ovate-oblong. Importantly, this lobe surpasses the tube in length, possesses a keel, and features an inwardly turned tooth. The corolla itself can vary in colour, appearing greenish or orange-yellow, and it is densely woolly and short-hairy on both sides. Within the flower, the corona lobes are fleshy and relatively tiny in size. The anther exhibits a distinct curvature over the stigmatic head, forming a short gynostegium. Additionally, the ovary of *L. reticulata* is positioned superiorly. Transitioning to the fruit, it takes the form of a pair of slender, ovoid to oblong follicles, which are sub-woody and turgid. These follicles measure approximately 6 to 8 cm in length and 2 to 2.5 cm in width, and they possess a tapering to blunt apex. Importantly, the fruit is glabrous and contains numerous seeds. The seeds themselves are ovoid and flattened, characterized by the presence of a coma a cluster of long, silky hairs situated at one end. These unique botanical characteristics collectively define *L. reticulata* as a distinctive and noteworthy plant species.

L. reticulata experiences rapid growth from April to September, with flowering occurring from May to July and fruit formation from November to February. Seed

maturation is from December to May, and germination occurs from June to September. The habit of *L. reticulata* (Retz.) Wight & Arn. is illustrated in Figure 1.

1.2.5. Molecular and phytochemical studies

Characterizing wild germplasm, particularly in endangered plant species, is pivotal for establishing foundational data that aid in crafting strategies for their protection, conservation, and sustainable use.[36, 51] This procedure requires an accurate assessment of their genetic diversity using impartial indices and parameters.[52-53] Molecular genetic markers, inheritable traits of plants, play a significant role in identifying and assessing genetic parameters within populations.[40, 54] Molecular genetic approaches are therefore essential to management and conservation programs. Genetic diversity, marked by variations in gene or allele compositions or frequencies among individuals within a species or population, is a critical factor. Understanding the existing diversity in forest genetic resources is a prerequisite for designing effective forest gene conservation programs.[55] Populations or species with numerous individuals but limited genetic variation are more vulnerable to extinction than those with rich genetic diversity. Before the 1960s, plant genetics and breeding experiments primarily utilized markers derived from genes that controlled distinct phenotypes, such as morphological markers, for easy visual identification. These included traits like dwarfism, chlorophyll deficiencies, and variations in flower, seed, or leaf colour and morphology, assessed through provenance and progeny trials. However, these markers, limited in number and heavily influenced by environmental factors, exhibited low levels of polymorphism and were expressed only at the whole plant level.[56] The advent of DNA markers has revolutionized this

field, offering a more precise way to characterize genotypes and measure genetic relationships.[57]

Among the various PCR-based markers, RAPD and ISSR markers stand out for their efficiency and cost-effectiveness. These techniques require only a minimal amount of DNA for rapid polymorphism analysis and are straightforward to execute, without necessitating prior DNA sequence knowledge.[58, 59] RAPD and ISSR markers, two molecular typing methods, have been instrumental in detecting variation among plant species.[58, 60] A robust genetic marker for genetic variation studies should exhibit high genetic variability and the capacity to generate multiple loci data from the genome.[61] ISSR markers, compared to RAPD, offer greater reproducibility and are more economical than AFLP, making them ideal for a variety of studies, including those on genetic variability and diversity, DNA fingerprinting[62], and phylogenetic analysis.[63] Differentiating closely related cultivars is facilitated by the ISSR method's ability to generate more intricate marker patterns than the RAPD approach.[64, 65] Additionally, ISSR markers, owing to their longer primers that anneal to a microsatellite sequence, allow for higher annealing temperatures, enhancing their specificity and reproducibility compared to RAPD markers.[66] These two techniques have been employed in combination for various plant species, such as *Commiphora wightii*[67], *Eucalyptus tereticornis*[68], *Jatropha curcas*[69], *Tectona grandis*[70], *Thunbergia coccinea*[71], *Picrorhiza kurroa*[72], etc., to investigate their genetic diversity.

However, there is a notable gap in the population genetic study of *L. reticulata*, particularly concerning the existing variations. While some research using RAPD markers on *L. reticulata* has been conducted[73], the application of ISSR markers for this species remains unexplored. ISSR markers have been widely used to study population

genetics in various plant species, aiding in the identification of different species, cultivars, or populations and in selecting genotypes/populations for breeding programs.[74-77]

It is known that *L. reticulata* contains a wide variety of therapeutically active chemicals including triterpenoids, leptadenol, n-tricontane, cetyl alcohol, β-sitosterol, α-amyrin acetate, lupanol 3-O diglucoside, leptidin, luteolin, diosmetin, stigmasterol, and α-tocopherol. Analytical techniques including TLC and HPTLC have verified the presence of quercetin and isoquercetin in the dried aqueous extract of the whole *L. reticulata* plant.[27] To detect α-amyrin, β-sitosterol, lupeol, and n-triacontane in the methanolic extract of *L. reticulata*, a unique HPTLC method has been created.[78] Furthermore, p-coumaric acid in *L. reticulata* extracts has been quantified using an accurate and verified HPLC method.[79]

In a different investigation, HPLC was used to identify polyphenols including rutin, quercetin, and p-coumaric acid in the ethyl acetate extract of *L. reticulata* leaves.[11] To determine the amount of rutin in *L. reticulata* leaves, an effective and specific HPTLC method has also been created and validated.[78] Furthermore, 31 phytocomponents were identified by gas chromatography-mass spectrometry (GC–MS) examination of the ethanolic entire plant extract of *L. reticulata*.[80] Subsequent GC–MS analysis of eight different extracts disclosed 77 phytochemicals, including γ-sitosterol, campesterol, pristane, hexahydrofarnesol, stearic acid, arachidic acid, coniferyl alcohol, n-tetracosanol-1, ascorbic acid 2,6-dihexadecanoate, and (2S,3S)-3,7,4'-Trihydroxy-5-methoxy-6-methylflavanone in *L. reticulata*. These compounds are recognized for their biological activity and pharmacological significance.[81] The variation in metabolite composition among different plants can be attributed to various eco-geographical

factors, including climatic conditions, soil characteristics, and the presence of specific microbial communities.[82] Numerous global studies have focused on the variability of phytochemicals in plants of industrial importance.[82-85] However, there is currently no documented utilization of HPTLC techniques for the analysis of quercetin in *L. reticulata* samples collected from various regions of India.

In recent times, the limitations of traditional morphological and chemical methods in authenticating plant products have become evident, paving the way for the development of novel methodologies in the quality control of botanicals. Molecular techniques such as Sequence Characterized Amplified Region (SCAR), derived from RAPD amplicons, are increasingly used for accurate genotype identification, especially in Ayurvedic medicinal plant parts.[40, 86] For genetic analysis of populations, software like POPGENE, which is freely available, is commonly employed. Similar to this, microsatellite or Simple Sequence Repeat (SSR) markers are useful for breeding, informative, and affordable.[87-89] Plants play a crucial role in human life, not only as a source of agricultural and horticultural products but also in the development of medicinal properties. Consequently, the conservation of plant diversity has become a global concern. Ex-situ conservation involves protecting endangered species outside their natural habitat,[90] while in-situ conservation methods are essential for maintaining biodiversity within natural ecosystems. Unfortunately, in-situ conservation is not always feasible due to human-induced habitat degradation and fragmentation, leading to the threat of extinction for many species.[91] In such scenarios, ex-situ conservation, especially through botanical gardens, becomes vital for preserving plant species.[90, 92]

The demand for plant based products is on the rise, partly due to their diverse properties and commercial value, with raw materials like *L. reticulata* fetching significant market

prices (INR 211/kg).[1] The scarcity and high cost of these plants often lead to their adulteration, which can be unintentional contamination or deliberate substitution with similar species. *L. reticulata* is now listed as endangered by the Wildlife Institute of India (WII) under the category of special habitats and threatened plants of India[73], is frequently substituted with plants like *W. volubilis* and *H. ada-kodien*, which bear a resemblance to it.[4, 93-96]

Numerous methods for assessing the quality and verifying the authenticity of herbal plants have been developed.[97, 98] DNA barcoding stands out as a powerful technique, used either independently or alongside conventional methods. This approach overcomes the challenges associated with morphological and physiological characteristics, enabling species verification without the need for in-depth taxonomic knowledge. DNA barcoding utilizes a specific segment of the genome, known as the "DNA barcode,"[99] which may be derived from the chloroplast, mitochondrial, or nuclear genome, and is employed for species identification at the molecular level.[100] In the field of herbal identification, this technology is revolutionizing the classification of herbal materials and is expected to have a major impact on the return of taxonomic methodologies.[101] The internal transcribed spacer 2 (ITS2) region has been recognized as the primary DNA barcode for the authentication of medicinal plants.[102] This ITS2 region is widely used in the identification of these plants.[102, 103]

Despite its advantages, DNA barcoding in herbal medicine faces challenges, including labour-intensive and expensive post-PCR processes, which can be a barrier in less developed countries. Recent studies have brought to light an innovative approach, high-resolution melting (HRM), for identifying herbal medicines. Research indicates that HRM, when combined with DNA barcoding markers, is highly effective in

differentiating medicinal plants and detecting falsification in commercially available herbal products.[104-106] HRM technology differentiates nucleic acid samples based on their melting temperature, employing direct melting curves to identify slight variations in PCR-amplified sequences. These variations are identified using DNA-specific dyes, cutting-edge equipment, and complex analysis software. The differentiation of samples is based on factors such as their composition, length, guanine-cytosine (GC) content, and nucleotide strands.[107] One of the benefits of HRM is its capability for immediate analysis following amplification, rendering it suitable for medium to high-throughput scenarios. In our study, we utilize the ITS2 barcode in combination with HRM technology to precisely identify *L. reticulata* and its adulterants, thereby improving the quality control and management of herbal products within the pharmaceutical supply chain.

1.3. Need for the study

As was previously mentioned, the woody climber *L. reticulata*, also known as Jivanti, was once ubiquitous in Rajasthan, Gujarat, Punjab, the Himalayas, the Khasi Hills, Sikkim, the Deccan Plateau, the Konkan Hills, Karnataka, and the Kerala region. It flourished at elevations up to 2000 meters.[16, 19] This plant occupies a significant position in traditional medicinal practices, renowned for its rejuvenating, revitalizing, and lactogenic properties.[3] Jivanti is a crucial ingredient in a variety of herbal preparations such as Speman, Envirocare, Calshakti, Antisept, and Chyawanprash, playing a substantial role in the market for herbal medicines. However, excessive harvesting has severely reduced its wild populations, leading the Wildlife Institute of India to categorize *L. reticulata* as a species at risk of extinction.[2, 73]

The growing discrepancy between demand and supply has led to increased incidents of adulteration and substitution, undermining the credibility and effectiveness of the herbal drug industry. Misidentification, substitution, and adulteration of *L. reticulata* have become widespread issues, with common adulterants including *W. volubilis* and *H. ada-kodien*.[4] This situation necessitates urgent measures to halt the depletion of this vital medicinal plants and to develop strategies for its conservation and sustainable use. For this, an in-depth understanding of the species' current genetic and phytochemical diversity is necessary. Moreover, addressing the quality issues related to misidentification, substitution, and adulteration is crucial, and this requires the establishment of a standard DNA-based authentication tool for the accurate identification and detection of *L. reticulata*.

Furthermore, there is a significant gap in knowledge regarding the genetic diversity among *L. reticulata* populations and the corresponding variations in their

phytochemicals. Plants, especially those exploited for industrial purposes, are more vulnerable to rapid population decline than smaller plants. Unscientific collection practices, including the harvesting of immature or vital parts, exacerbate this issue. Additionally, a lack of adequate training and awareness among collectors and officials, coupled with the unethical exploitation of plant resources in a largely unregulated crude drug market, further complicates the situation. An understanding of genetic and phytochemical diversity, along with the development of identification tools, is crucial in addressing these challenges.

To formulate effective management and conservation strategies for this medically valuable plant species, it is imperative to understand the variations within and among its populations. Molecular techniques such as RAPD and ISSR based analysis are relatively simple, moderately sensitive, and widely used for genetic studies. For the quantification of chemical constituents, HPTLC has emerged as a precise, rapid, and cost-effective technique, often considered the standard for estimating phytoconstituents. Therefore, a combination of genetic and phytochemical profiling is a powerful approach to understanding diversity and informing conservation efforts. In this study, ISSR, RAPD analysis, and HPTLC quantification of selected phytochemicals were performed on fifty-five samples from eleven populations of *L. reticulata* collected from different regions of India.

Methods utilizing DNA have become increasingly favoured for the definitive identification of plant species, as DNA is present in all tissues and remains unaffected by environmental or physiological influences.[108, 109] Consequently, the combination of DNA barcoding and High-Resolution Melting (HRM) analysis emerges as a precise, dependable, swift, economical, and sturdy technique for differentiating *L.*

reticulata from its common adulterants. The primary objective of this study is to guide stakeholders in developing strategies for the effective preservation of *L. reticulata*, as well as for its identification and distinction from frequently used substitutes or adulterants in the context of quality assurance.

1.4. Objectives

- To study the DNA profile of *Leptadenia reticulata,* from different geographical regions of India using ISSR/RAPD primers

- To investigate the phytoconstituent variation in *Leptadenia reticulata* using a fingerprinting method

- To develop the molecular markers for identification and differentiation of *Leptadenia reticulata* from its adulterants/substituents

Chapter- 2
Materials and Methods

2. Materials and Methods

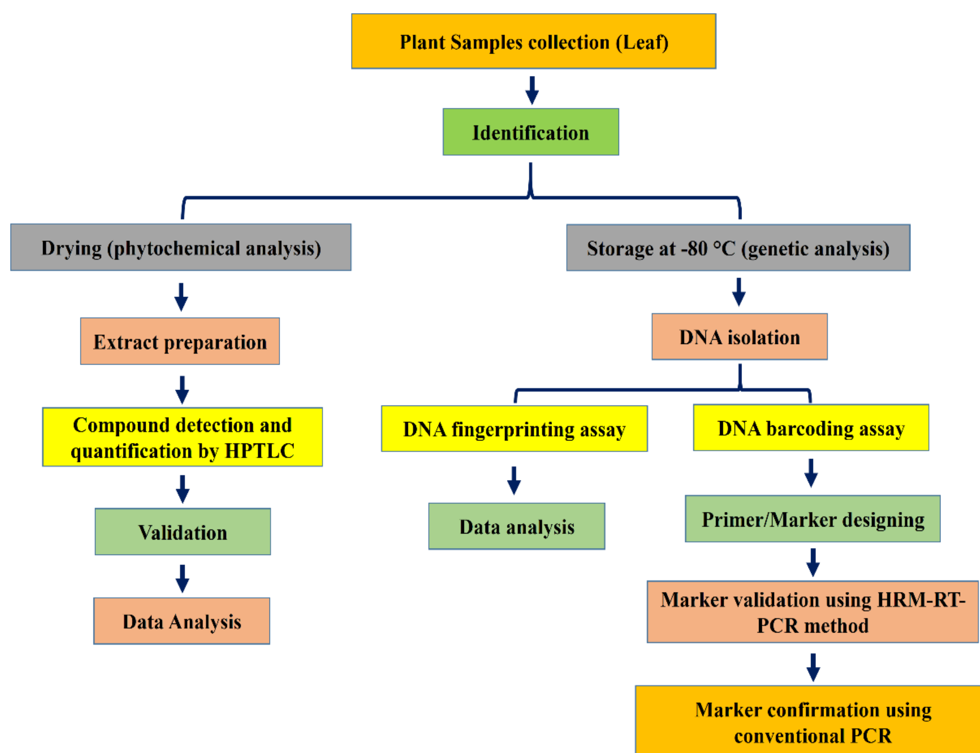
2.1. List of materials and chemicals required in the project

S. No	Material Name	Procurement Source
1	CTAB	Himedia
2.	Tris base	Himedia
3.	EDTA	Merck, India
4.	Chloroform	Merck, India
5.	Isoamyl alcohol	Merck, India
6.	PEG	Merck, India
7.	Ethanol	China
8.	RNase	Genei^{TM}, Bangalore
9.	Primers (ISSR, RAPD and ITS2)	IDT, USA
10.	PCR components	Genei^{TM}, Bangalore
11.	DNA ladder	Genei^{TM}, Bangalore
12.	EtBr dye	Genei^{TM}, Bangalore
13.	DNA loading dye	Genei^{TM}, Bangalore
14.	Agarose	Genei^{TM}, Bangalore
15.	Quercetin	Fluka, USA
16.	Methanol	Merck, India
17.	Toluene	Merck, India
18.	Ethyl acetate	Merck, India
19.	Formic acid	Merck, India
20.	Acetic acid	Merck, India
21.	Pre-coated TLC silica gel plates	Merck, Germany

2.2. List of instruments used in this project

S. No.	Instruments Name	Company Details
1.	Biophotomoter	Eppendorf AG, 22331, Hamburg, Germany
2.	Centrifuge	Eppendorf AG, 5424R, Germany
3.	Mastercycler	Eppendorf AG, nexus GX2, Hamburg, Germany
4.	Gel electrophoresis unit	BIO-RAD, PowerPac^{TM} Basic, Singapore
5.	Gel documentation system	Syngene G: Box F3, UK
6.	Real-Time PCR system	Thermo Fisher Scientific, Applied Biosystems, Singapore
7	DNA Analyzer	Thermofisher Scientific, ABI 3730XL 96 capillary, Singapore
8.	HPTLC instrument	*CAMAG*, TLC Scanner 4, Switzerland
9.	Incubator	KEMI, KIS.3FDT, Kerala, India
10.	pH meter	BUTECH INSTRUMENTS, Singapore
11.	Shaker incubator	Eppendorf, New Brunswick^{TM}, USA
12.	Digital weight balance	RADWAG, AS 220.R2 PLUS, Poland
13.	Oven	KEMI, KOMS.3FDT, Kerala, India
14.	Refrigerator	BLUE STAR, CHF 425, Mumbai
15.	Sonicator	BRANSONIC series, CPX1800H-E, USA
16.	Spinwin	MC-00, TARSON, India
17.	Water bath	META-LAB Scientific Industries, Mumbai

2.3. Flow chart for the study work



2.4. To study the DNA profile of *Leptadenia reticulata,* from different geographical regions of India using ISSR/RAPD primers

2.4.1. Collection and identification of plant samples

In this study, Fifty-five *L. reticulata* samples were collected from eleven different geographical locations in India and separately stored at −20°C for genetic study analysis (Figure 2 and Table 1). *Leptadenia reticulata* (Retz.) Wight & Arn. with voucher number RMRC-1647 was deposited to the ICMR-NITM herbarium. The gene region encoding ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) was sequenced from the voucher specimen and deposited in GenBank (Accession number: OR885730). A laboratory identifying code was provided to each sample and preserved for further study.

Figure 2: Geographical distribution of *L. reticulata* accessions from different regions.

[**BP**: Bhatinda, Punjab; **BR**: Barmer, Rajasthan; **JDRJ**: JNVU - Jodhpur, Rajasthan; **AJRJ**: AFRI - Jodhpur, Rajasthan; **KRJ**: Kota, Rajasthan; **JG**: Junagarh, Gujarat; **DG**: Diyodar, Gujarat; **AJG**: Agri - Junagarh, Gujarat; **KMP**: Khandwa, Madhya Pradesh; **JUP**: Jhansi, Uttar Pradesh; **BK**: Belgaum, Karnataka (Source: Google Map)]

Table 1: Sample collection details.

S. No	Location	Location code	Sample size	Latitude (N)	Longitude (E)
1.	Bathinda, Punjab	BP	09	30.2110°	74.9455°
2.	Junagadh, Gujarat	JG	06	21.5222°	70.4579°
3.	Diyodar, Gujarat	DG	04	24.1095°	71.7771°
4.	Khandwa, Madhya Pradesh	KMP	04	21.8314°	76.3498°
5.	Barmer, Rajasthan	BR	11	25.7521°	71.3967°
6.	AFRI-Jodhpur, Rajasthan	AJRJ	06	26.2389°	73.0243°
7.	Kota, Rajasthan	KRJ	04	25.2138°	75.8648°
8.	Jhansi, Uttar Pradesh	JUP	02	25.4484°	78.5685°
9.	JNVU-Jodhpur, Rajasthan	JDRJ	04	26.2440°	73.0206°
10.	Belgaum, Karnataka	BK	03	15.8497°	74.4977°
11.	Agriculture-Junagadh, Gujarat	AJG	02	21.5063°	70.4502°

2.4.2. Genomic DNA Extraction

Following the modified Cetyl Trimethyl Ammonium Bromide method (CTAB), DNA was extracted from all collected *L. reticulata* leaf samples.[110, 111] CTAB method is a cationic detergent with solubilized plant cell wall and lipid membrane of internal organelles and denature proteins and enzymes thus it prevents the hydrolysis of DNA during its isolation. Initially, the plant sample was ground with the help of liquid N_2 to avoid tissue damage followed by an additional of CTAB buffer to the ground plant tissue. After centrifugation, the supernatant was treated with Chloroform: Isoamyl alcohol to separate proteins from the DNA. The DNA was then precipitated using

ammonium acetate and chilled absolute ethanol. The DNA pellet was washed with 70% ethanol and dissolved in 1X TE buffer before being stored.[110, 112]

2.4.3. Quantitative and Qualitative measurement of DNA

Further, the quantity and purity of DNA were measured using Eppendorf Biophotometer (Hamburg, Germany) set to 260/280 nm[113] and visualized under UV light (Syngene G: Box F3, UK) using 0.8% agarose gels stained with EtBr (GeNei™, India). The DNA samples have been preserved at -20 °C for future study use.

2.4.4. DNA fingerprinting assays using ISSR and RAPD markers

ISSR and RAPD assays were carried out to fingerprint all plant DNA samples. 15 ISSR (University of British Colombia, IDT) and 10 RAPD (OP, Series, IDT) were procured for amplification of DNA samples of *L. reticulata*. Depending upon consistency and band clarity, suitable primer pairs were selected for additional experiments. Cycling conditions for carrying out ISSR (Table 2) and RAPD (Table 3) assays were optimized.[114]

Table 2: Reaction condition for ISSR-PCR amplifications.

	PCR condition			
ISSR primer	Step	Tem.	Time	Cycle
UBC-Series	Initial denaturation	94°C	5 minutes	1
	Denaturation	94°C	30 seconds	
	Annealing	50°C	45 seconds	45
	Extension	72°C	1 minutes	
	Final extension	72°C	5 minutes	1
	Holding	4°C	∞	-

Table 3: Reaction condition for RAPD-PCR amplifications.

RAPD primer	PCR condition Step	Tem.	Time	Cycle
	Initial denaturation	94°C	3 minutes	1
OP-Series	Denaturation	94°C	45 seconds	
	Annealing	50°C	30 seconds	35
	Extension	72°C	1 minutes	
	Final extension	72°C	7 minutes	1
	Holding	4°C	∞	-

Both ISSR and RAPD PCR amplification were accomplished in volume of 25µL mixtures comprising 40ng template, 200µM of each dNTP (GeNei™, India), 10µM primer, 0.5µL Taq polymerase (3U/µL; GeNei™, India), along with buffer supplied by the manufacturer (Tris HCl, pH 9.0; 15mM $MgCl_2$). The amplifications were carried out in a Mastercycler® Nexus GX2 (Eppendorf, Germany). The amplified PCR samples were visualized after running agarose gels (i.e., 1.5%) in buffer (1X TAE) and staining using Gel loading dye (GeNei™, India). The Gel Documentation System (Syngene G: Box F3, UK) was utilized for viewing and capturing gel photographs. Controls were maintained without addition of DNA as template.

2.4.5. Genetic data analysis

For *L. reticulata* samples, the ISSR bands were scored based on their presence (1) or absence (0). Total bands, polymorphic bands and their percentages were calculated for each primer. The Polymorphic Information Content (PIC) was derived using the

equation 1-p2-q2, where 'p' denotes the frequency of present bands and 'q' indicates the frequency of absent bands.[115] The Marker Index (MI) was then ascertained by the product of the Effective Multiplex Ratio (EMR) and PIC (MI = EMR×PIC).[116] Various genetic variation indicators were evaluated, including the percentage of polymorphic bands (PPB), observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), Nei's genetic diversity (h), total heterozygosity (H_T) within population heterozygosity (H_S), Nei's genetic differentiation index among populations (G_{ST}), and gene flow estimates among populations (Nm). These were calculated using POPGENE version 1.31.[117] An analysis of molecular variance (AMOVA) was performed with GenALEx version 6.5, aiming to examine genetic variation both within and across populations.[118] Principal Coordinate Analysis (PCoA) was conducted to assess the genetic distances among individuals and to confirm the consistency of genetic differentiation among populations as indicated by cluster analysis, utilizing GenALEx version 6.5.[118] Moreover, Nei's genetic distance was used to create a matrix of genetic distance and identity. The creation of an unweighted pair group method with arithmetic mean (UPGMA) dendrogram, which was essential for examining population variance, was made by this matrix. NTSYS-pc v. 2.02 and POPGENE version 1.31 were used in this procedure.[117, 119]

2.5. To investigate the phytoconstituent variation in *Leptadenia reticulata* using fingerprinting method

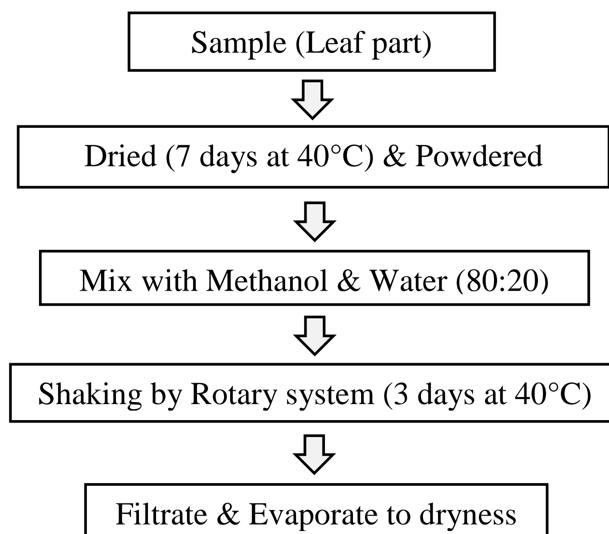
2.5.1. Collection of samples and extraction

L. reticulata samples were collected from five different geographical locations across India, with each site's GPS coordinates meticulously recorded (Table 4). The collected samples were subjected to shade drying before being finely pulverized. Each powdered sample was then processed according to the method outlined in Schematic Diagram 1. In the following stage, 15 g of *L. reticulata* powder was mixed with 255 mL of 80% methanol (Merck Life Science, Mumbai) and stirred on a rotary shaker (New Brunswick™, USA) for 72 hours at a standard room temperature (37°C ± 3). Subsequently, the mixture was filtered using Whatman no. 1 filter paper, evaporated in a water bath at 40°C, and preserved at 4°C for later use, as indicated in Schematic Diagram 1.[120, 121]

Table 4: Plant sampling.

S. No	Sample code	Cultivation region	Latitude (N)	Longitude (E)
1.	HT	Hyderabad (Telangana)	17.3850°	78.4867°
2.	DG	Deodar (Gujarat)	24.1095°	71.7771°
3.	BK	Belagavi (Karnataka)	15.8497°	74.4977°
4.	BP	Bathinda (Punjab)	30.2110°	74.9455°
5.	BR	Baran (Rajasthan)	25.2474°	77.1528°

Schematic diagram 1: Extract preparation



2.5.2. Standard and Sample preparation

Quercetin (98% purity) was sourced from Fluka (USA) (see Figure 3). A Quercetin standard solution was formulated at a 1 mg/mL concentration in a glass volumetric flask. Before applying this solution on a Thin Layer Chromatography (TLC) plate, it was first filtered through Whatman no. 1 filter paper. Similarly, the extract was prepared at a 10 mg/mL concentration and filtered using Whatman no. 1 filter paper for the purpose of analytical study.

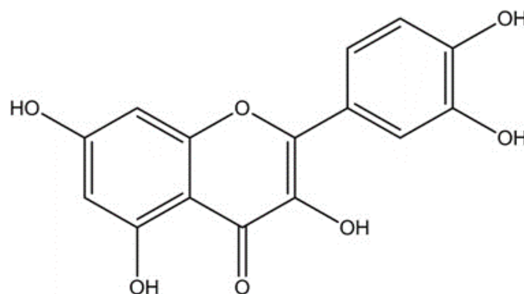


Figure 3: Molecular structure of quercetin.

2.5.3. HPTLC Apparatus and Chromatographic Conditions

The HPTLC technique was established based on a method previously documented for extracts.[122] Utilizing a *CAMAG* Hamilton microliter syringe and a Linomat V (*CAMAG*, Muttenz, Switzerland), samples were applied as 8mm bands on a TLC silica gel aluminium plate. The slit dimensions were set at 6×0.45mm, with a scanning speed consistently held at 20mm/s. Toluene, chloroform, ethyl acetate, and formic acid were added in a volumetric ratio of 2.5:2:4.5:1 to prepare the mobile phase. The plates underwent development in a *CAMAG* twin trough chamber, pre-saturated with the mobile phase for about 20 minutes. The samples were allowed to run a distance of 7 cm in a controlled environment at 25°C ± 2 and 60% relative humidity. After development, the plates were left to air dry and were then examined at 300 nm using a *CAMAG* TLC scanner IV equipped with vision cats-software (version 3.1), using deuterium light as the radiation source. The composition of the mobile phase was fine-tuned for optimal resolution, and the Retention factor (R_f) value for quercetin was established at 0.49. The chosen 300 nm wavelength matched the maximum absorption point of the quercetin spot.

2.5.4. Validation of HPTLC Method

The method was validated following ICH guidelines to confirm its appropriateness for the intended application.[123] method validation involved examining the method's accuracy, reliability, and consistency. Key parameters including linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and recovery rates were thoroughly investigated.[123-124]

2.5.4.1. Linearity

For linearity assessment, a quercetin standard solution was prepared in methanol at 40 µg/mL (from 1mg/mL stock solution). Quercetin was injected in triplicate in various volumes (1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 µL, n = 6) on a TLC plate, providing a concentration range of 60–160 ng/spot. A regression equation was generated by analysing the connection between peak area and concentration using a linear least squares regression analysis.

2.5.4.2. LOD and LOQ

LOD and LOQ were established using a specific formula.[123]

This is expressed as: LOD = 3XN/B;

LOQ = 10XN/B

Here, N denotes the standard deviation of the area of the standard (n = 6 for noise calculation), and B represents the slope of the calibration curve.

2.5.4.3. Precision and Accuracy

The precision (inter-day and intra-day), and accuracy (recovery) were determined for the developed method. For intra-day evaluation, triplicate analyses of samples at 80, 100, and 120 ng/spot concentrations were conducted (n = 6). The inter-day precision and accuracy were then determined by repeating the intra-day tests over three days. Precision at each level was calculated as the coefficient of variation (CV, %) for the respective concentrations. Accuracy was determined by calculating the percentage recovery of a known added quantity.[123]

2.5.4.4. Specificity

To confirm the method's specificity, the standard solution's band was analysed. This involved comparing the R_f values and the spectra of the band with those of the standard quercetin solution, verifying quercetin's presence in the samples. Further developed chromatograms and plates are scanned and checked for overlap in the spectra and R_f value.

2.5.4.5. Robustness

The method's robustness was tested by slightly altering chromatographic conditions, such as the mobile phase saturation time, composition, and volume. These modifications were limited to ensure less than a 2% variation in the %RSD (Relative Standard Deviation) parameters, confirming the method's stability under varied conditions.

2.6. To develop the molecular markers for identification and differentiation of *Leptadenia reticulata* from its adulterants/substitutes

2.6.1. ITS2 Marker-Based DNA Barcoding

L. reticulata, commonly known as Jivanti, and its adulterants, including *W. volubilis* and *H. ada-kodien*, were distinguished using the ITS2-based DNA barcoding technique. For this purpose, DNA samples of *L. reticulata* (one sample from each of 11 localities) and samples of its adulterants *W. volubilis* (one sample from each of 5 localities) and *H. ada-kodien* (one sample from each of 4 localities) were used (Table 5). *L. reticulata* and its adulterants were submitted to herbariums at ICMR-NITM, Belagavi, and CCRAS, Chennai, India (Figure 4; Table 6).

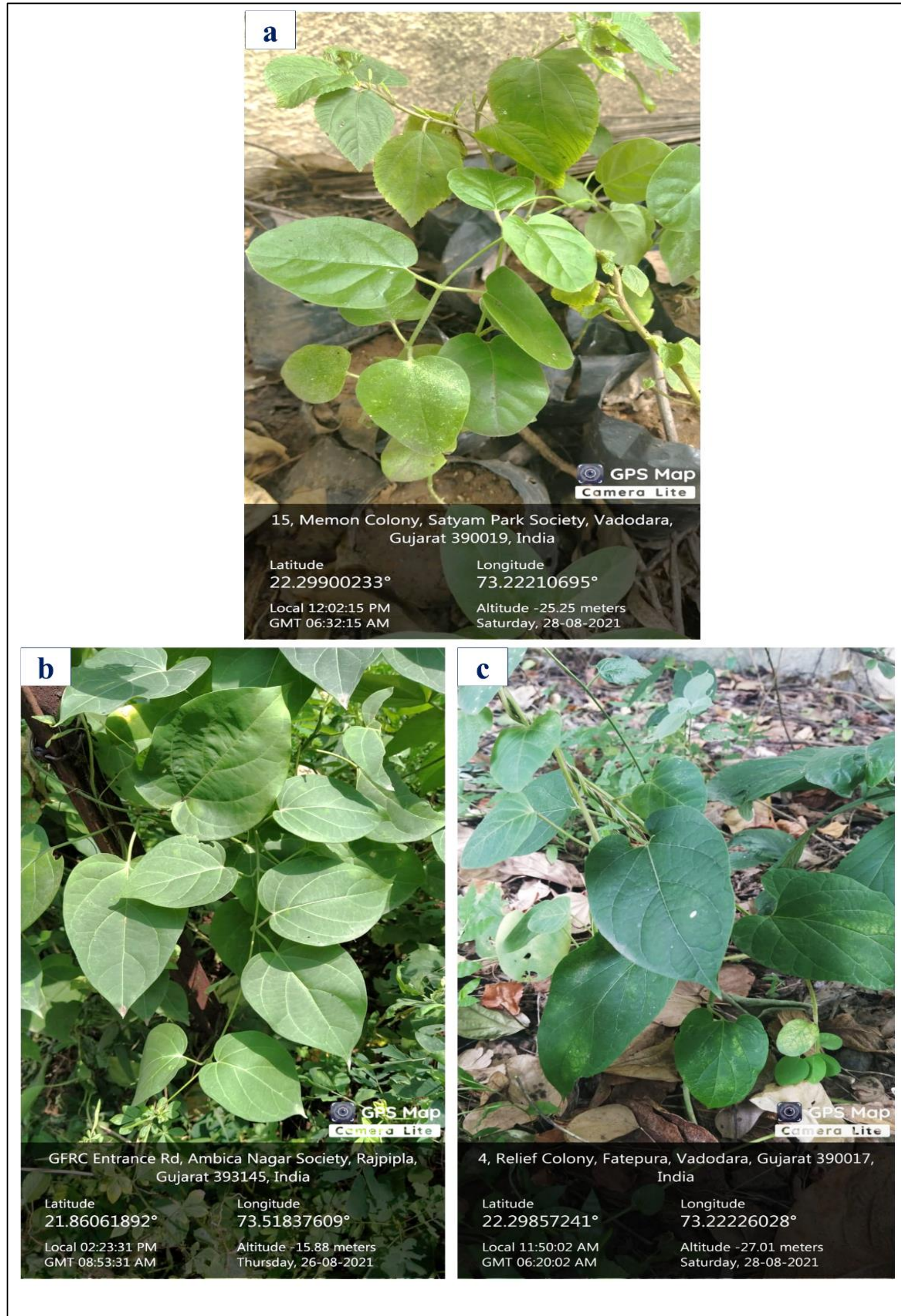


Figure 4: Samples habitat. (a): *Leptadenia reticulata;* (b): *Wattakaka volubilis;* (c): *Holostemma ada-kodien*

Table 5: Collection of *Leptadenia reticulata* and its adulterations.

S. No	Plant name	Location	Sample code	Latitude (N)	Longitude (E)
1.	*L. reticulata*	Khandwa, MP	LR-1	21.8314°	76.3498°
2.	*L. reticulata*	AFRI - Jodhpur, RJ	LR-2	26.2389°	73.0243°
3.	*L. reticulata*	KVK- Barmer, RJ	LR-3	25.7521°	71.3967°
4.	*L. reticulata*	JNVU - Jodhpur, RJ	LR-4	26.2440°	73.0206°
5.	*L. reticulata*	Jhansi, UP	LR-4	25.4484°	78.5685°
6.	*L. reticulata*	Diyodar, GJ	LR-5	24.1095°	71.7771°
7.	*L. reticulata*	Bhatinda, PB	LR-7	30.2110°	74.9455°
8.	*L. reticulata*	Junagarh, GJ	LR-8	21.5222°	70.4579°
9.	*L. reticulata*	Vadodara, GJ	LR-9	22.2990°	73.2221°
10.	*L. reticulata*	CAZRI - Jodhpur, RJ	LR10	26.2626°	72.9971°
11.	*L. reticulata*	Jamnagar, GJ	LR11	22.4707°	70.0577°
12.	*W. volubilis*	Dolatpur-Junagarh, GJ	WV-1	21.5221°	70.4578°
13.	*W. volubilis*	Rajpipla, DH	WV-2	21.8606°	73.5183°
14.	*W. volubilis*	Kakdwip- Kolkata, WB	WV-3	21.8760°	88.1853
15.	*W. volubilis*	Udupi, KA	WV-4	13.3409°	74.7421°
16.	*W. volubilis*	Gadag-KA	WV-5	15.4026°	75.6208°
17.	*H. ada-kodien*	Silvassa, DH	HAK-1	20.2753°	73.0073°
18.	*H. ada-kodien*	Vadodara, GJ	HAK-2	22.2985°	73.2222°
19.	*H. ada-kodien*	Nadapuram, KL	HAK-3	11.6840°	75.6552°
20.	*H. ada-kodien*	Chennai, TN	HAK-4	13.0827°	80.2707°

MP: Madhya Pradesh; RJ: Rajasthan; UP: Uttar Pradesh; GJ: Gujarat; PB: Punjab; DH: Dadra and Nagar Haveli; WB: West Bengal; KA: Karnataka; KL: Kerala; TN: Tamil Nadu

Table 6: Voucher specimen numbers.

S. No	Plant Name	Family	Voucher Number
1.	*Leptadenia reticulata* (Retz.) Wight & Arn.	Apocynaceae	RMRC-1647
2.	*Wattakaka volubilis* (L.f.) Stapf.	Apocynaceae	CSMCARI/PS/0239
3.	*Holostemma ada-kodien* Schult.	Apocynaceae	CSMCARI/PS/0237

2.6.2. DNA Extraction and ITS2-Based Amplification

A modified CTAB procedure was applied to extract genomic DNA from the collected plant samples. This process included samples from eleven of *L. reticulata*, five of *W. vobubilis*, and four of *H. adakodien,* where the ITS2 region were effectively amplified. To ensure clear band patterns suitable for sequencing, the ITS2 region was first optimized using gradient PCR. This amplification of the ITS2 region within the nuclear gene took place in an eppendorf thermal cycler. Each PCR mixture, with a total volume of 50µL, comprised 50ng of genomic DNA, 10µM of ITS2 primer, and 25µL of Taq Master Mix Red, which included 1.5Mm $MgCl_2$ (AMPLIQON). The primers used for the ITS2 region were ITS2 forward: ATGCGATACTTGGTGTGAAT, and ITS2 reverse: ACGCTTCTCCAGACTACAAT.[125] To enhance the PCR reaction, 100% DMSO was added to each mixture.[126] The thermal cycling protocol for ITS2 amplification began with an initial denaturation at 98°C for 5 minutes. This was followed by 40 cycles, each consisting of denaturation at 98°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 10 minutes.[51, 125] Using a 1.5% agarose gel in 1X TAE buffer solution, agarose gel electrophoresis was used to assess the PCR results. The electrophoresis of the PCR

products was conducted alongside a 100bp DNA ladder/marker at 60 volts for 120 minutes. The results were observed using a gel documentation system (Syngene G: Box F3, UK). Subsequently, all the amplified samples were prepared for further analysis.

2.6.3. High Resolution Melting (HRM) Curve Analysis

Primers were carefully designed based on sequences derived from ITS2, as outlined in Table 7. The DNA amplification process utilized the Step One Plus™ Real-Time PCR system from Applied Biosystems (Thermo Fisher Scientific Inc, USA), which was instrumental in creating distinct melting temperature (Tm) profiles for identification purposes. Each reaction mixture, with a total volume of 20µL, consisted of 10µL of iTaq Universal SYBR® Green Supermix dye (2X concentration, Bio-Rad, USA), 1µL of the DNA template, and 1µL of 0.1µM of both forward and reverse primers, sourced from IDT, USA. This was further augmented with 7µL of water free from RNase and DNase. A first denaturing step at 95°C for 2 minutes was followed by 39 cycles of 95°C for 15 seconds, 56°C for 10 seconds, and 72°C for 20 seconds in the real-time PCR. During the expansion stage, fluorescence data was collected. During the extension stage, fluorescence data was recorded. For HRM analysis, the PCR products were subjected to a final denaturation step at 95°C for 15 seconds. Subsequently, measuring the fluorescence intensity at each interval, melting curves were created by progressively raising the temperature by 0.3°C from 75°C to 95°C. The Step One Plus software (version 2.2) was utilized for the analysis of these peaks/curves.

Table 7: Primers used for HRM analysis.

Designed marker name	Sequence (5'- 3')	Direction	Size (bp)
LR01	GGGGCGGAATTTGGCTTC CACGAAGGGTCCGAGTGC	Forward Reverse	152
LR02	AATTTGGCTTCCCGTACCTC CGTTCGTGGACAGTTGCC	Forward Reverse	182
LR03	CGAGGTCCGTGGTAGAGC GAAGGGTCCGAGTGCTCT	Forward Reverse	172

2.6.4. Detection of *L. reticulata* by Conventional PCR

With a minor adjustments, the aforementioned real-time PCR assays were used to achieve PCR amplification (40 cycles). Utilizing GeneSys (Syngene G: Box F3, UK), the PCR products were observed and recorded after being separated on 1.5% agarose gels.

2.6.5. ITS2 Data Analysis

Sequence alignment and phylogenetic analysis, employing the maximum likelihood method, were conducted using MEGA software (version 7.0.14).[127]

Chapter- 3
Data Analysis Plan

3. Data Analysis plan

3.1. Genetic and phytochemical data

The ISSR and RAPD profiles were transformed into a binary matrix, which was then used for the assessment of population genetic parameters through POPGENE version 1.31.[117] The software GenAlEx 6.4 was instrumental in creating AMOVA and PCoA analyses. Furthermore, PCA was carried out with the support of BioVinci software (version 1.1.0) for Windows (BioTuring Inc., San Diego California, USA). Additionally, the Marker Index (MI) and Polymorphism Information Content (PIC) calculations were carried out.[115-116] Nei's genetic distance was used to compute an identity and genetic distance matrix for the purpose of evaluating population variance. Following that, this matrix was used in POPGENE version 1.31 and NTSYS-pc version 2.02 to create a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA).[117, 119] Samples were run using a triplicate Thin Layer Chromatography (TLC) process, with Mean ± SD findings displayed (n = 3).

3.2. Establishment of genetic markers for identification and differentiation

Sequence alignments were carried out using MEGA (version 7.0.14),[127] and manually selected primers were designed for amplification of DNA samples of *L. reticulata.*

Chapter- 4
Results

4. Results

4.1. To study the DNA profile of *Leptadenia reticulata,* from different geographical regions of India using ISSR/RAPD primers

4.1.1. Polymorphism

Fifteen ISSR and ten RAPD random primers were used in a preliminary test for the ISSR and RAPD assay tests. From these, nine ISSR and five RAPD primers were sorted based on the consistency and uniqueness of the band patterns. The resulting sample fingerprints obtained using these selected primers are illustrated in Figure 5 (a - i) and Figure 6 (a - e). In the ISSR assay, a total of 287 genetic loci were identified, while the RAPD assay revealed 135 loci, with both assays demonstrating 100% polymorphism.

During the ISSR assay, the polymorphic bands amplified by each primer pair varied, with a range from 26 (UBC-817) to 35 (UBC-818), and an average of 31.89 bands. The Polymorphism Information Content (PIC) values fluctuated between 0.23 (UBC-864) and 0.30 (UBC-826), with an average of 0.28. This suggests a moderate level of variability among the nine primers, as detailed in Table 8. The Marker Index (MI) values exhibited a range from 10.47 (UBC-834) to 7.12 (UBC-817), averaging at 8.82 (refer to Table 8).

In the context of the RAPD assay, the range of polymorphic bands for each primer pair was observed to be between 24 (OPD-3) and 30 (OPD-20), with an average of 27 bands. The PIC values here showed a variation from 0.16 (OPD-20) to 0.22 (OPA-3), averaging at 0.190, which again indicates a moderate level of variability among the primers (as shown in Table 9). The MI values in this assay were found to range from 5.64 (OPA-3) to 4.64 (OPD-3), with an overall average of 5.13 (Table 9).

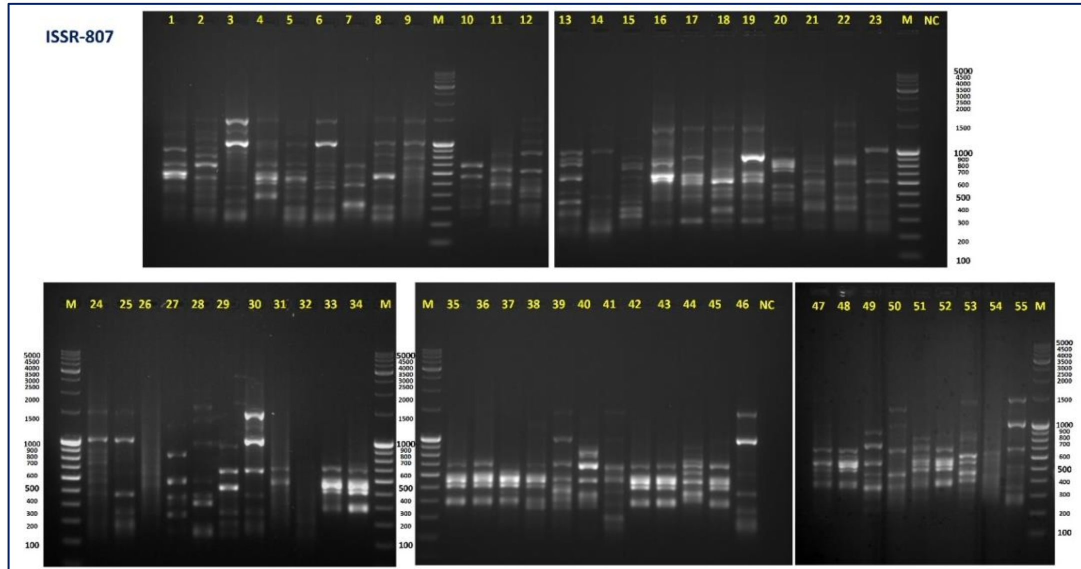


Figure 5 (a): ISSR profile of *L. rericulata* genotypes with primer UBC-807. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

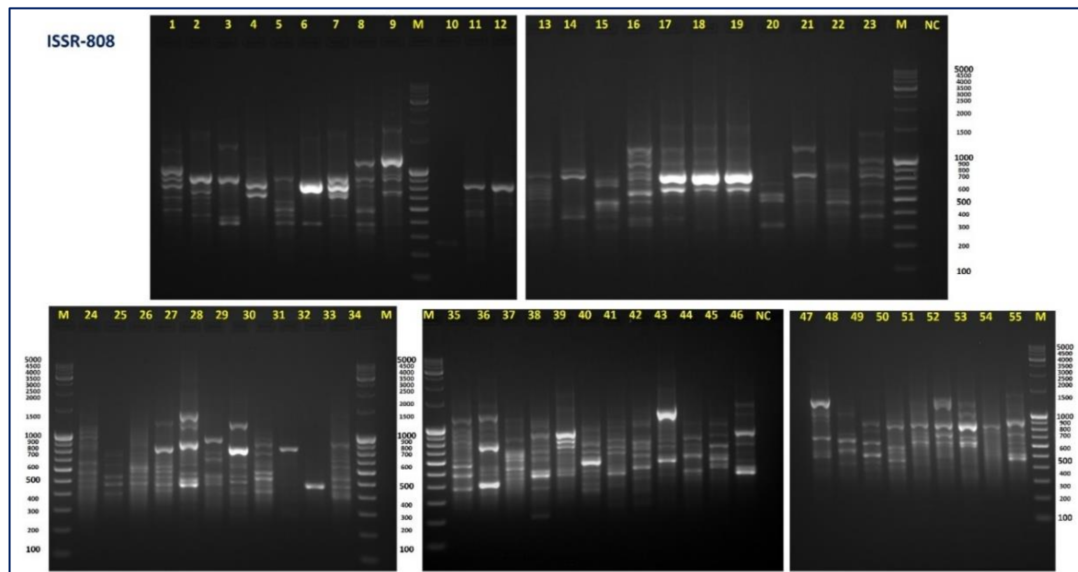


Figure 5 (b): ISSR profile of *L. rericulata* genotypes with primer UBC-808. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

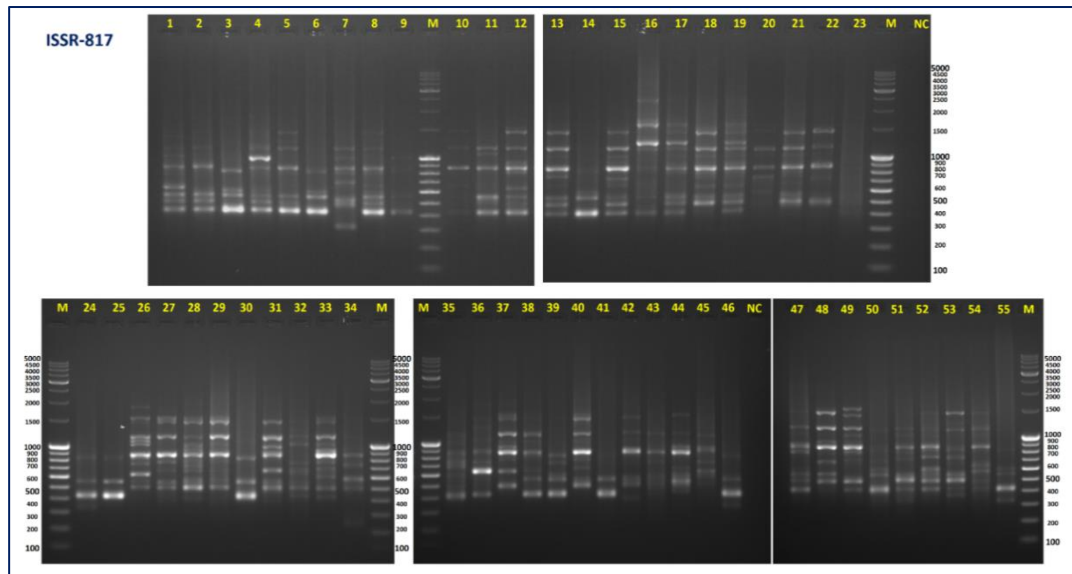


Figure 5 (c): ISSR profile of *L. rericulata* genotypes with primer UBC-817. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

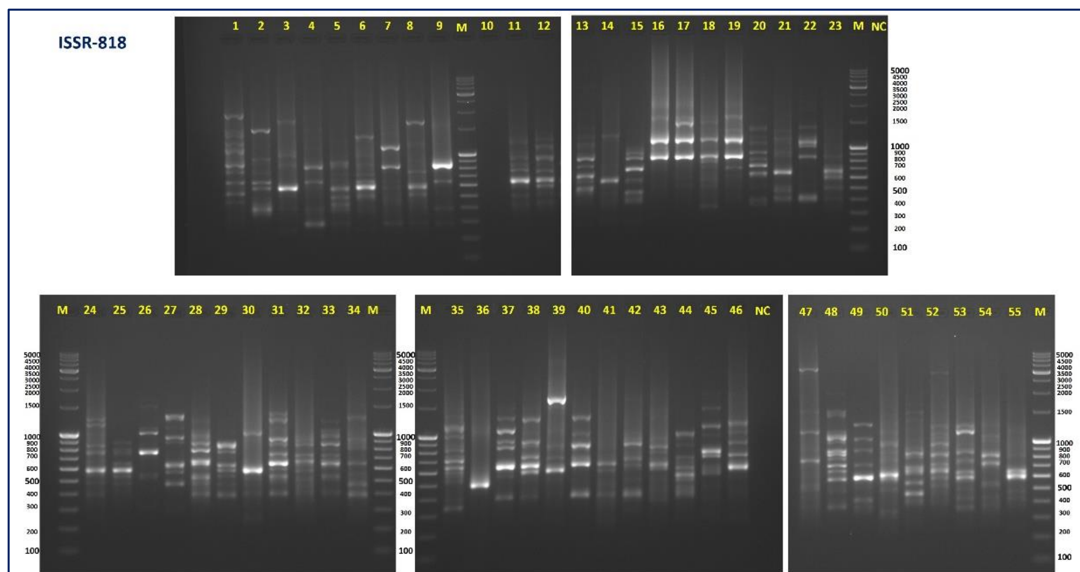


Figure 5 (d): ISSR profile of *L. rericulata* genotypes with primer UBC-818. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

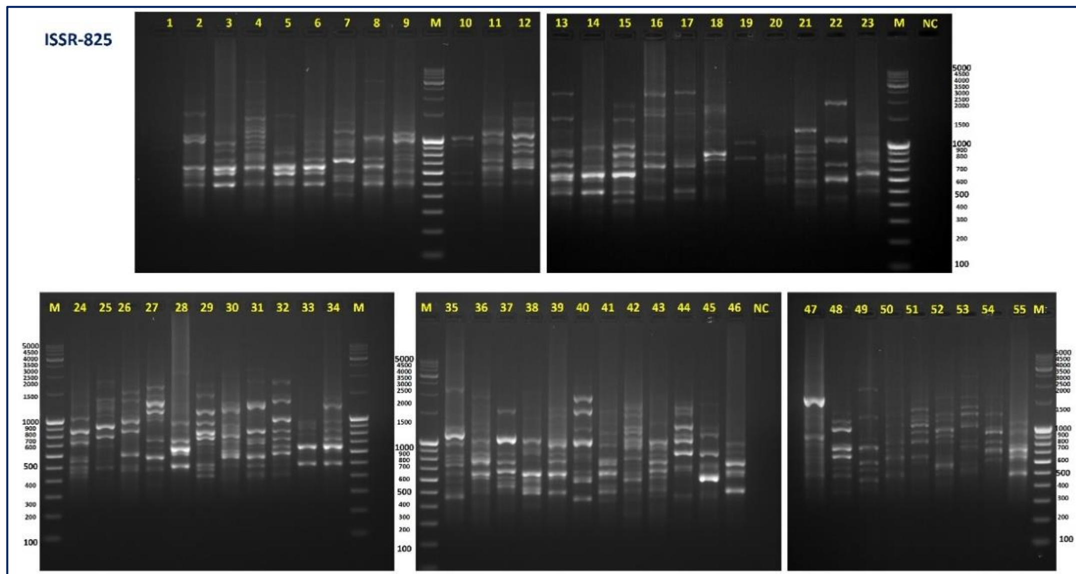


Figure 5 (e): ISSR profile of *L. rericulata* genotypes with primer UBC-825. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

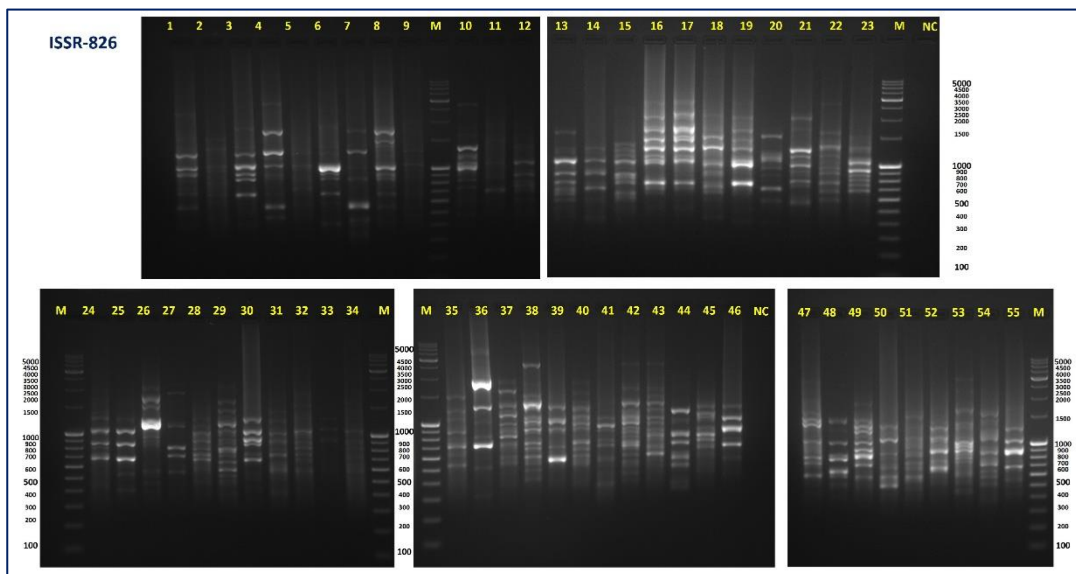


Figure 5 (f): ISSR profile of *L. rericulata* genotypes with primer UBC-826. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

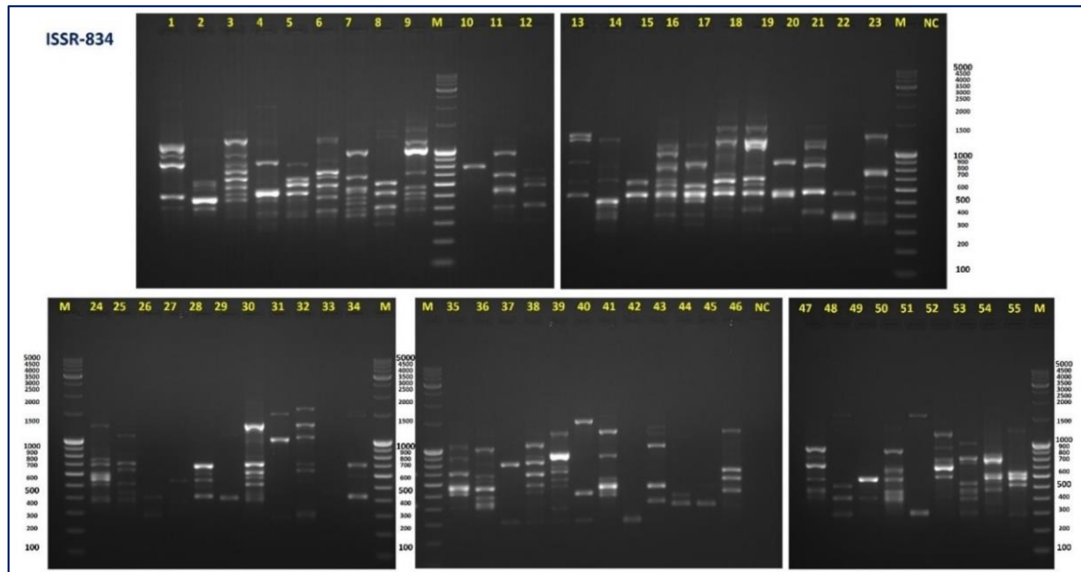


Figure 5 (g): ISSR profile of *L. rericulata* genotypes with primer UBC-834. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

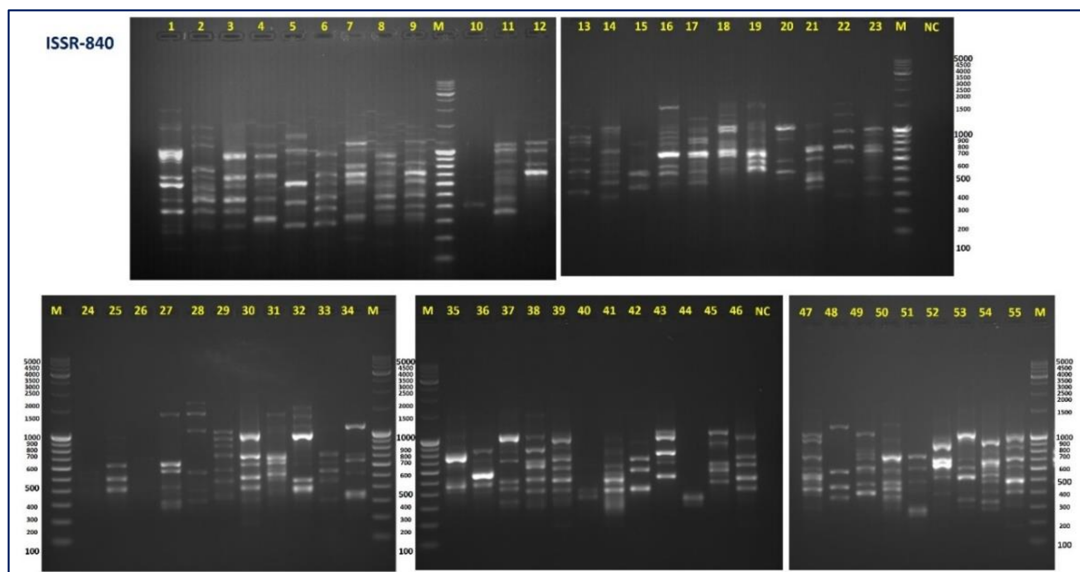


Figure 5 (h): ISSR profile of *L. rericulata* genotypes with primer UBC-840. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

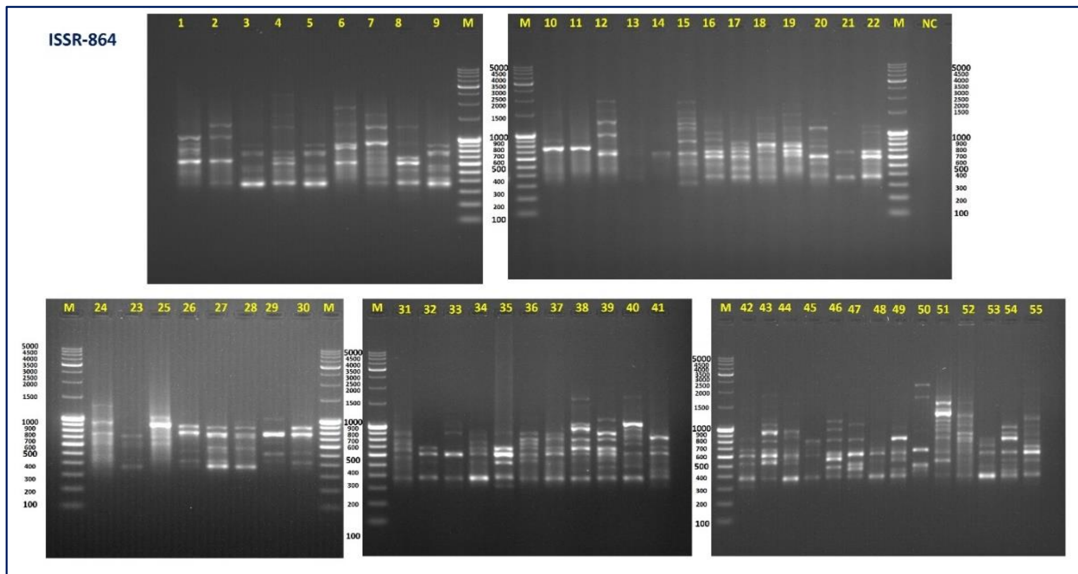


Figure 5 (i): ISSR profile of *L. rericulata* genotypes with primer UBC-864. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

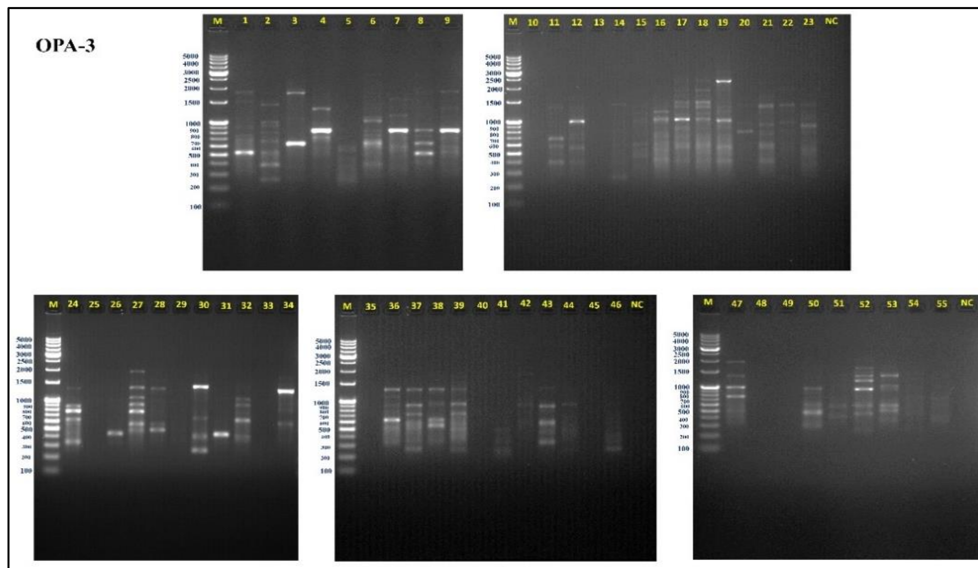


Figure 6 (a): RAPD profile of *L. rericulata* genotypes with primer OPA-3. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

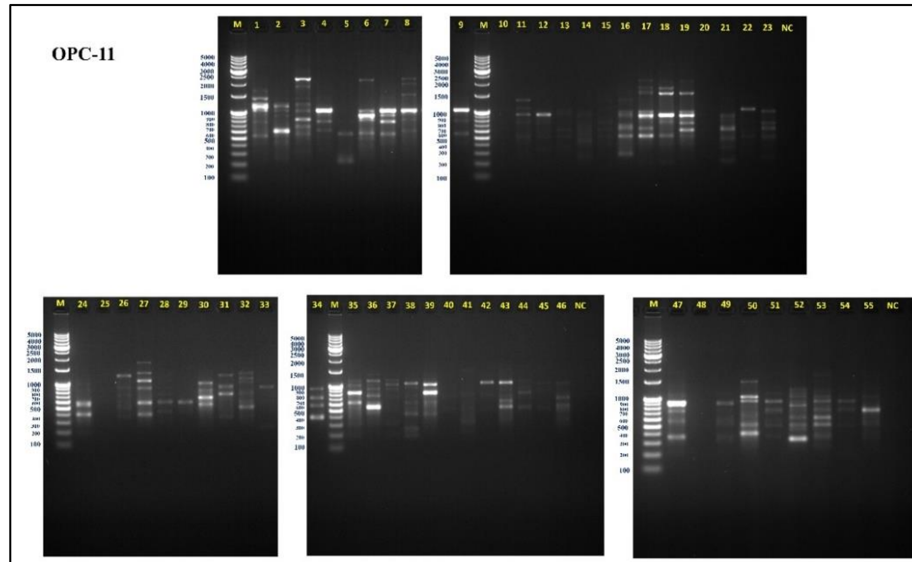


Figure 6 (b): RAPD profile of *L. rericulata* genotypes with primer OPC-11. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

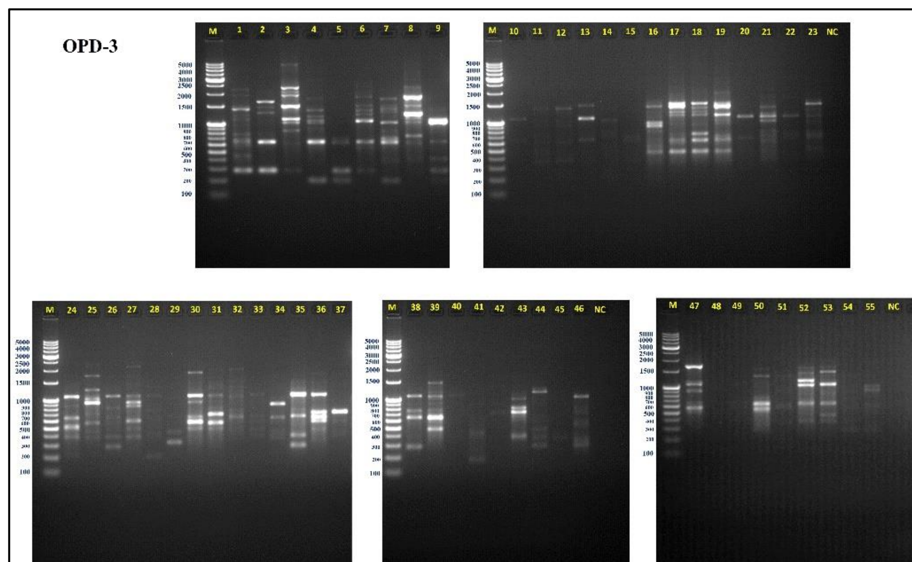


Figure 6 (c): RAPD profile of *L. rericulata* genotypes with primer OPD-3. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

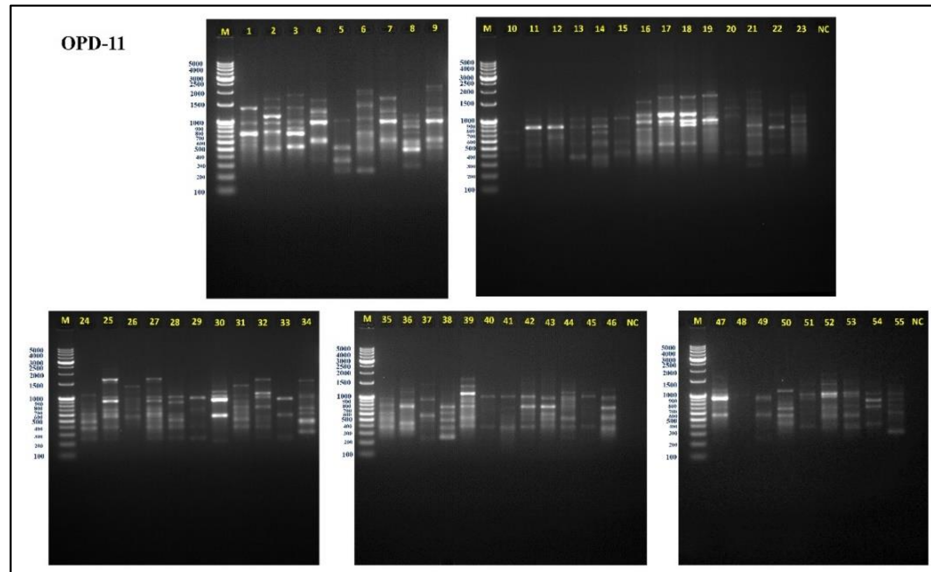


Figure 6 (d): RAPD profile of *L. rericulata* genotypes with primer OPD-11. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

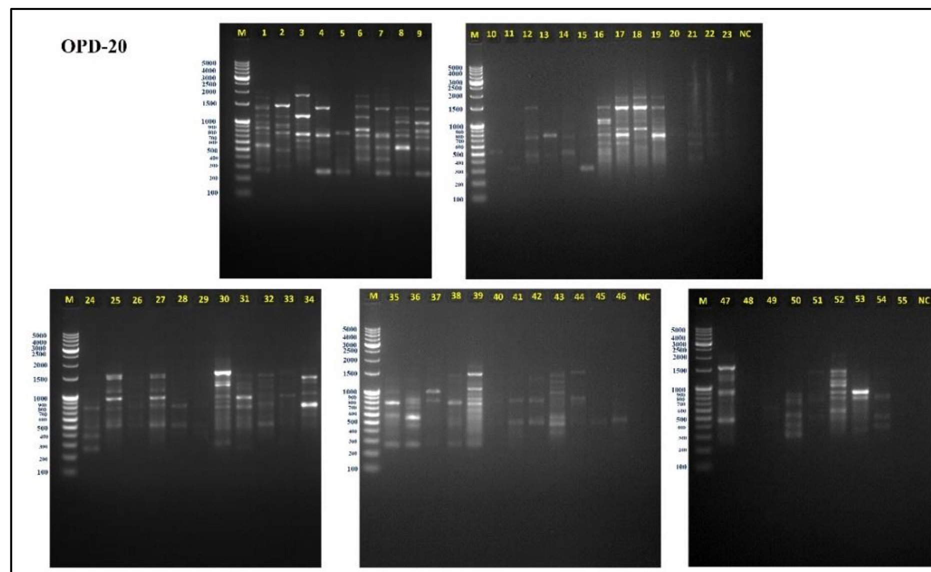


Figure 6 (e): RAPD profile of *L. rericulata* genotypes with primer OPD-20. Lanes 1-55: correspond to the *L. reticulata*; Lane M: DNA size marker (100+500bp).

Table 8: Details of the nine ISSR markers selected for genetic diversity of *Leptadenia reticulata* populations.

S. No	Marker	Sequence (5'-3')	TB	PB	PB %	PIC	EMR	MI
1.	UBC-817	CAC ACA CAC ACA CA	26	26	100	0.30	26	7.80
2.	UBC-818	CAC ACA CAC ACA CG	35	35	100	0.26	35	9.06
3.	UBC-825	ACA CAC ACA CAC A	34	34	100	0.27	34	9.21
4.	UBC-826	ACA CAC ACA CAC AC	33	33	100	0.30	33	10.02
5.	UBC-834	AGA GAG AGA GAG AG	35	35	100	0.29	35	10.47
6.	UBC-807	AGA GAG AGA GAG A	28	28	100	0.29	28	7.89
7.	UBC-808	AGA GAG AGA GAG A	33	33	100	0.28	33	9.19
8.	UBC-840	GAG AGA GAG AGA GA	32	32	100	0.27	32	8.66
9.	UBC-864	CACACACACACACACART	31	31	100	0.23	31	7.12
Total				287		2.49		79.43
Average				31.89		0.283		8.825

Here, TB = Total number of Amplified bands; PB = Number of polymorphic bands; PB% = Percentage polymorphic bands; PIC = Polymorphic Information Content; EMR= Effective Multiplex Ratio; MI = Marker Index

Table 9: Details of the nine RAPD markers selected for genetic diversity of *Leptadenia reticulata* populations.

S. No	Marker	Sequence (5'-3')	TB	PB	PB %	PIC	EMR	MI
1.	OPA-3	AGTCAGCCAC	26	26	100	0.22	26	5.64
2.	OPC-11	AAAGCTGCGG	27	27	100	0.19	27	5.32
3.	OPD-3	GTCGCCGTCA	24	24	100	0.19	24	4.64
4.	OPD-11	AGCGCCATTG	28	28	100	0.17	28	5.01
5.	OPD-20	ACCCGGTCAC	30	30	100	0.16	30	5.03
Total				135		0.95		25.66
Average				27		0.19		5.13

Here, TB = Total number of Amplified bands; PB = Number of polymorphic bands; PB% = Percentage polymorphic bands; PIC = Polymorphic Information Content; EMR= Effective Multiplex Ratio; MI = Marker Index

4.1.2. Genetic diversity

To determine the variables affecting genetic diversity among 11 distinct locations of *L. reticulata*, a thorough investigation was conducted. As indicated in Table 10, the average observed allele count (*N*a) was found to be 1.47, with a variation (standard deviation, SD) of ± 0.479. This figure fluctuated between 1.19 in the JUP population and 1.70 in the BP population. The average effective number of alleles (*N*e) per locus was determined to be 1.32 (SD ± 0.381), spanning from 1.19 (JUP) to 1.39 (BP). The Shannon's information index (*I*) showed variation from 0.13 (JUP) to 0.35 (BR), with a mean value of 0.27 (SD ± 0.292). Nei's gene diversity (*h*) was found to vary between 0.13 (BR) and 0.23 (AJG), with an overall average of 0.18 (SD ± 0.204). The highest percentage of polymorphic loci was observed in the BP population at 69.7%, while the lowest was 19.5%, with an overall average of 46.8%. In line with Hardy-Weinberg equilibrium expectations, the estimated heterozygosity (H_S) across all populations (H_T) was 0.27 (SD ± 0.022) and 0.18 (SD ± 0.010) for pooled populations (H_S). The average genetic differentiation (G_{ST}) and gene flow (*N*m) were calculated to be 0.32 and 1.05, respectively. Analysis through AMOVA revealed that 89% of the genetic variation occurred within populations, and 11% occurred between populations, as depicted in Figure 7. These findings suggest that these groups have a considerable degree of genetic diversity.

Table 10: Genetic diversity parameters for 55 samples from 11 locations of *L. reticulata* using ISSR markers.

S. No	Lab code	N	*Na*	*Ne*	*h*	*I*	P (%)
1.	BP 1-9	200	1.697 ± 0.480	1.381 ± 0.339	0.232 ± 0.181	0.354 ± 0.259	69.69
2.	JG 1-6	157	1.547 ± 0.499	1.345 ± 0.361	0.204 ± 0.198	0.305 ± 0.288	54.70
3.	DG 1-4	119	1.415 ± 0.493	1.302 ± 0.379	0.172 ± 0.208	0.251 ± 0.301	41.46
4.	KPM 1-4	132	1.459 ± 0.499	1.340 ± 0.391	0.193 ± 0.213	0.279 ± 0.307	45.99
5.	BR 1-11	197	1.686 ± 0.465	1.397 ± 0.366	0.235 ± 0.192	0.354 ± 0.271	68.64
6.	AJRJ 1-6	161	1.561 ±0.497	1.369 ± 0.376	0.215 ± 0.203	0.318 ± 0.292	56.1
7.	KRJ 1-4	120	1.418 ± 0.494	1.299 ± 0.373	0.172 ± 0.203	0.251 ± 0.299	41.81
8.	JUP 1-2	56	1.195 ± 0.397	1.195 ± 0.397	0.098 ± 0.199	0.135 ± 0.275	19.51
9.	JDRJ 1-4	134	1.467 ± 0.499	1.329 ± 0.372	0.190 ± 0.207	0.279 ± 0.301	46.69
10.	BK 1-3	123	1.436 ± 0.497	1.348 ± 0.397	0.194 ± 0.221	0.277 ± 0.316	43.55
11.	AJG 1-2	77	1.268 ± 0.444	1.268 ± 0.444	0.134 ± 0.222	0.186 ± 0.308	26.83
	Average		1.468 ± 0.477	1.324 ± 0.381	0.185 ± 0.204	0.272 ± 0.292	46.81
	At species level		1.989 ± 0.101	1.439 ± 0.303	0.274 ± 0.149	0.428 ± 0.193	98.97

Here, *Na* = Observed number of alleles; *Ne* = Effective number of alleles; *h* = Nei's genetic diversity; *I* = Shannon's Information Index; N = Number of polymorphic loci; P % = Percentage of polymorphic loci.

Table 11: Diversity indices for *L. reticulata* samples generated by ISSR analysis.

S. No	*N*	H_T	H_S	G_{ST}	*Nm*
1.	55	0.273	0.185	0.322	1.055

Here, N = Number of samples; H_T = Total heterozygosity; H_S = within population heterozygosity; G_{ST} = Genetic differentiation; *N*m = Gene flow

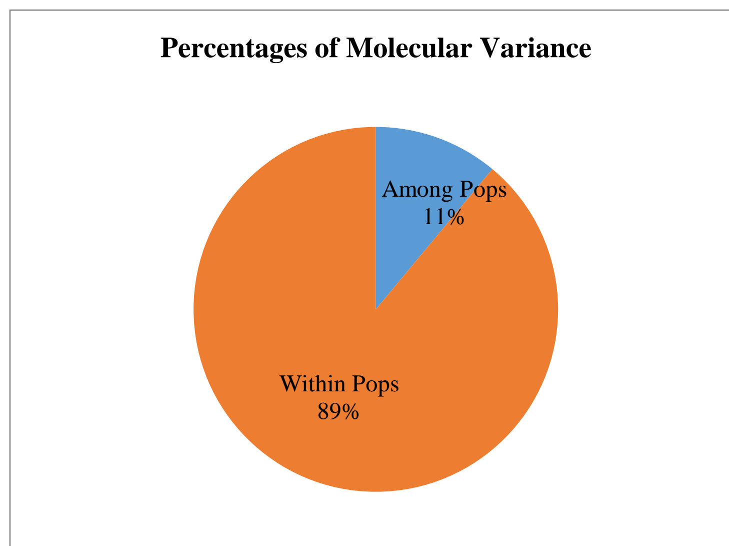


Figure 7: ISSR markers based AMOVA.

Subsequent examinations were conducted on the same 11 populations of *L. reticulata*, as outlined in Table 12. In this analysis, the average observed allele count (*N*a) was marginally lower, at 1.32 (SD ± 0.416), and ranged from 1.06 (in the JUP location) to 1.78 (in the BP location). The average effective allele number (*N*e) per locus was calculated to be 1.19 (SD ± 0.288), spanning from 1.06 (JUP) to 1.39 (BP). The mean value of Shannon's information index (*I*) showed variation, with the lowest being 0.04

(JUP) and the highest 0.38 (BR), and an average of 0.17 (SD ± 0.243). The range of Nei's gene diversity (*h*) was observed from 0.03 (JUP) to 0.25 (BP), with a mean value of 0.11 (SD ± 0.167). Notably, the BP population exhibited the highest proportion of polymorphic loci at 78.5%, while the lowest was 6.6%, with an overall average of 32.5%. The calculated heterozygosity across all locations (H_T) was found to be 0.17 (SD ± 0.012), and within other location (H_S), it was 0.11 (SD ± 0.005). The estimated mean genetic differentiation (G_{ST}) and gene flow (*N*m) stood at 0.32 and 1.04, respectively, as indicated in Table 13. Analysis of Molecular Variance (AMOVA) demonstrated that 97% of the variation occurred within populations, and 3% between populations, as depicted in Figure 8. These findings suggest a moderately high level of genetic diversity observed within 11 *L. reticulata* locations.

Table 12: Genetic diversity parameters for 55 samples from 11 locations of *Leptadenia reticulata* using RAPD markers.

S. No	Lab code	N	*N*a	*N*e	*h*	*I*	P (%)
1.	BP 1-9	106	1.785±0.412	1.398±0.316	0.248±0.165	0.383±0.233	78.52
2.	JG 1-6	33	1.244±0.434	1.129±0.250	0.081±0.150	0.125±0.226	24.44
3.	DG 1-4	47	1.348±0.478	1.253±0.365	0.144±0.201	0.210±0.291	34.81
4.	KPM 1-4	28	1.207±0.407	1.136±0.275	0.081±0.161	0.120±0.237	20.74
5.	BR 1-11	80	1.592±0.493	1.244±0.244	0.162±0.160	0.259±0.239	59.26
6.	AJRJ 1-6	54	1.400±0.491	1.230±0.315	0.141±0.182	0.214±0.270	40.0
7.	KRJ 1-4	32	1.237±0.426	1.165±0.310	0.096±0.175	0.141±0.255	23.70
8.	JUP 1-2	9	1.066±0.250	1.066±0.250	0.033±0.125	0.046±0.173	6.67
9.	JDRJ 1-4	42	1.311±0.464	1.204±0.315	0.122±0.184	0.180±0.271	31.11
10.	BK 1-3	43	1.318±0.467	1.254±0.274	0.141±0.207	0.202±0.297	31.85
11.	AJG 1-2	10	1.074±0.262	1.074±0.262	0.037±0.131	0.051±0.182	7.41
	Average		1.325±0.416	1.195±0.288	0.116±0.167	0.175±0.243	32.59
	At species level		2.000±0.000	1.259±0.186	0.190±0.109	0.325±0.148	88.31

Here, *Na* = Observed number of alleles; *Ne* = Effective number of alleles; *h* = Nei's genetic diversity; I = Shannon's Information Index; N = Number of polymorphic loci; P % = Percentage of polymorphic loci.

Table 13: Diversity indices for *Leptadenia reticulata* samples generated by RAPD analysis.

S. No	*N*	H_T	H_S	G_{ST}	*Nm*
1.	55	0.174	0.117	0.324	1.040

Here, N = Number of samples; H_T = Total heterozygosity; H_S = within population heterozygosity; G_{ST} = Genetic differentiation; *N*m = Gene flow

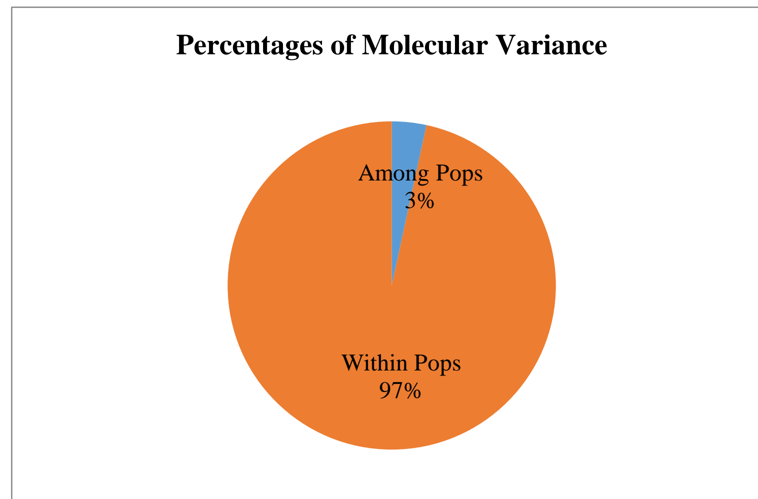


Figure 8: RAPD markers based AMOVA.

4.1.3. Cluster analysis

The ISSR-based analysis was employed to determine the genetic connections among 55 *L. reticulata* accessions using Jaccard's similarity coefficient. The highest species level genetic similarity was found to be 0.44 (between DG1 and DG2), while the lowest was 0.04 (between KRJ1 and KMP4). A dendrogram (UPGMA) constructed using Jaccard's similarity coefficient divided the 55 *L. reticulata* genotypes into two

primary clusters, as depicted in Figure 9. The first major group (A) contained 53 accessions, while the second minor cluster (B) included only two accessions from Belagavi, Karnataka (BK2 and BK3). Further subdivision resulted in sub-clusters A1 and A2 from the first main cluster, with A1 comprising four accessions (DG1, DG2, DG3, and DG4) and A2 containing 49 accessions. Cluster B showed the most genetic similarity among the Gujarat populations. Genotypes from Punjab (BP1, BP2, and BP5) and Karnataka (BK2 and BK3) were found to be more genetically distant from other genotypes. Nei's genetic distance among populations ranged from 0.035 (AJRJ-KRJ) to 0.164 (JUP-DG), as detailed in Table 14. The dendrogram generated two main clades, with all populations included in clade one, except for the DG population, which exhibited the highest difference among the populations (Figure 10). The UPGMA dendrogram, based on Nei's genetic distances (Table 14), showed the highest and lowest similarity between KRJ and AJRJ and JUP and DG, respectively.

In the RAPD-based analysis, the genetic relationships among 55 accessions of *L. reticulata* were assessed using Jaccard's similarity coefficient derived from scoring data. The resulting dendrogram, constructed via UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using Jaccard's coefficient, categorized the 55 *L. reticulata* genotypes into two primary clusters (illustrated in Figure 11). The first major group, Cluster A, encompassed 54 accessions, whereas the second, smaller Cluster B, included just one accession from Bhantida, Punjab (BP2). Further subdivision of Cluster A resulted in two sub-clusters: Sub-cluster A1 with four accessions and Sub-cluster A2 comprising 49 accessions. Interestingly, Cluster B displayed the highest genetic similarity with genotypes from Gujarat. The genotypes from Punjab and Karnataka were notably more genetically distinct from other genotypes, as depicted in Figure 11. Nei's genetic distance among the populations

varied, ranging from a low of 0.018 (between BR and JG) to a high of 0.150 (between AJG and DG), as detailed in Table 15. The dendrogram formed two main clades, with all populations falling into the first clade, except for the DG population, which exhibited the greatest genetic divergence (Figure 12). The largest and lowest genetic similarities between AJG and DG and BR and JG, respectively, were indicated by the UPGMA dendrogram, which was based on Nei's genetic distances (Table 15).

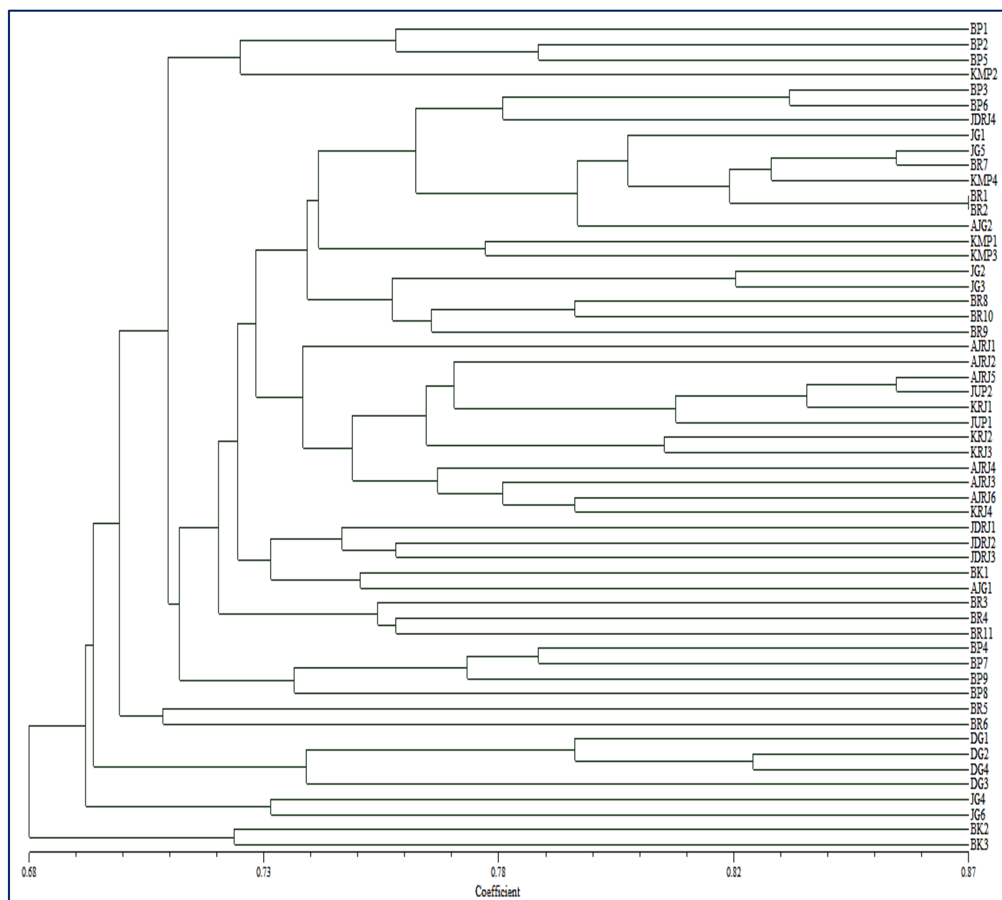


Figure 9: Phylogenetic relationship between 55 *Leptadenia reticulata* accessions represented by a dendrogram (UPGMA, NTSYS) based on ISSR marker genetic similarity matrix data (see Table 1 for code location).

Table 14: Population genetic distance: genetic distance below the diagonal and Nei's genetic identity above.

Pop	BP	JG	DG	KMP	BR	AJRJ	KRJ	JUP	JDRJ	BK	AJG
BP	****	0.9452	0.8854	0.9343	0.9375	0.9322	0.9235	0.9067	0.9309	0.9049	0.9049
JG	0.0564	****	0.8997	0.9515	0.9587	0.9148	0.9202	0.9112	0.9362	0.9028	0.9175
DG	0.1217	0.1057	****	0.8919	0.8983	0.866	0.8696	0.8482	0.8817	0.8566	0.8623
KMP	0.0680	0.0498	0.1144	****	0.9477	0.9209	0.9144	0.9094	0.9188	0.8795	0.8893
BR	0.0646	0.0422	0.1073	0.0537	****	0.9283	0.9272	0.9128	0.9259	0.9044	0.9248
AJRJ	0.0702	0.089	0.1439	0.0824	0.0744	****	0.9652	0.9322	0.9225	0.9049	0.9126
KRJ	0.0796	0.0831	0.1397	0.0894	0.0756	**0.0354**	****	0.9409	0.9223	0.9032	0.8998
JUP	0.0980	0.093	**0.1647**	0.0949	0.0912	0.0702	0.0609	****	0.8985	0.8897	0.8848
JDRJ	0.0716	0.066	0.1259	0.0847	0.0770	0.0807	0.0809	0.1070	****	0.9012	0.9096
BK	0.0999	0.1023	0.1547	0.1284	0.1005	0.0999	0.1018	0.1169	0.0821	****	0.8857
AJG	0.0999	0.0861	0.1482	0.1174	0.0781	0.0915	0.1056	0.1230	0.0948	0.1213	****

BP: Bhatinda, Punjab; BR: Barmer, Rajasthan; JDRJ: JNVU - Jodhpur, Rajasthan; AJRJ: AFRI - Jodhpur, Rajasthan; KRJ: Kota, Rajasthan; JG: Junagarh, Gujarat; DG: Diyodar, Gujarat; AJG: Agri - Junagarh, Gujarat; KMP: Khandwa, Madhya Pradesh; JUP: Jhansi, Uttar Pradesh; BK: Belgaum, Karnataka

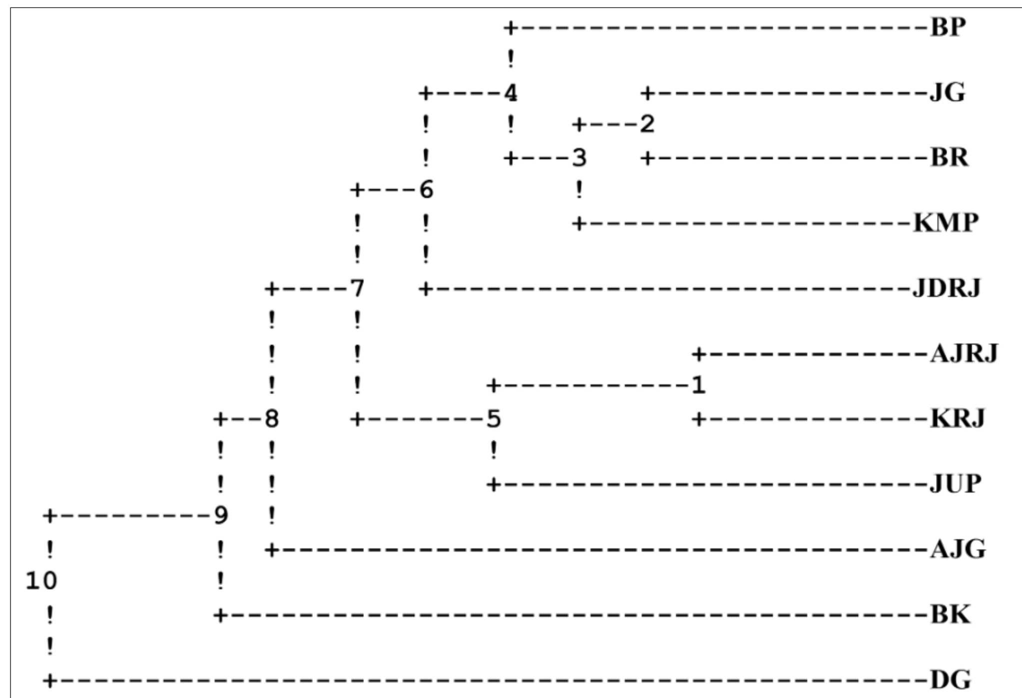


Figure 10: UPGMA based dendrogram of Nei's genetic identity of location wise *Leptadenia reticulata* samples (see Table 1 for location code).

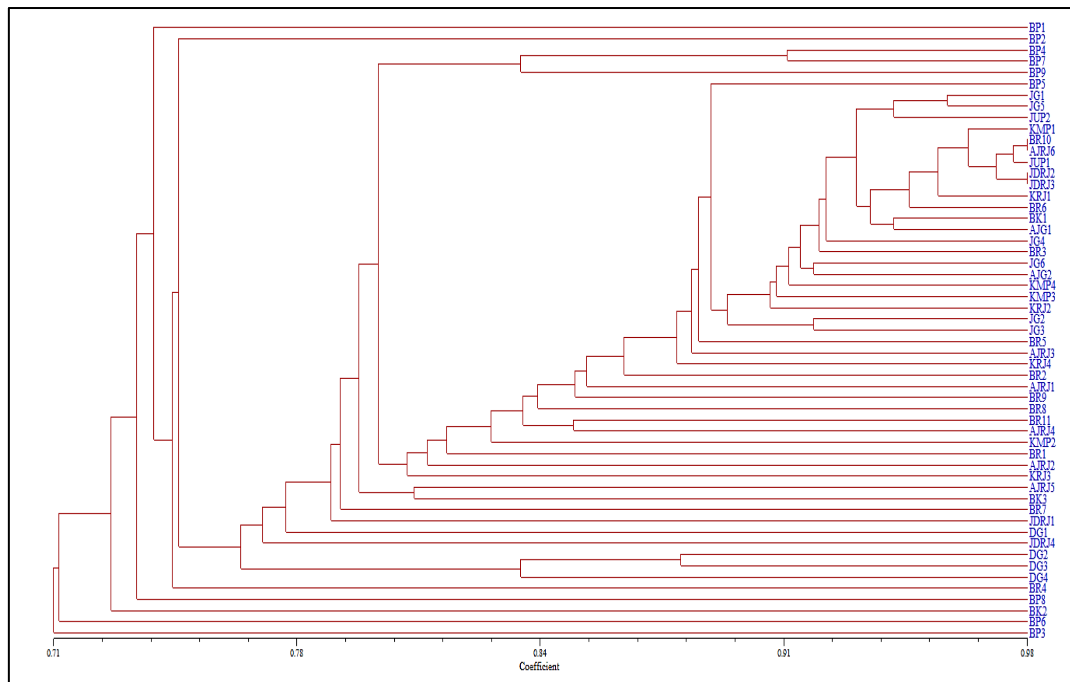


Figure 11: Phylogenetic relationship between 55 *Leptadenia reticulata* accessions represented by a dendrogram (UPGMA, NTSYS) based on RAPD marker genetic similarity matrix data (see Table 1 for code location).

Table 15: Population genetic distance: genetic distance below the diagonal and Nei's genetic identity above.

Pop	BP	JG	DG	KMP	BR	AJRJ	KRJ	JUP	JDRJ	BK	AJG
BP	****	0.9599	0.8934	0.9488	0.9554	0.9547	0.9446	0.9489	0.9468	0.9428	0.9341
JG	0.0409	****	0.8962	0.9781	0.9814	0.9628	0.9661	0.9794	0.9770	0.9490	0.9726
DG	0.1127	0.1096	****	0.8885	0.9011	0.9066	0.8873	0.8811	0.8882	0.8987	0.8606
KMP	0.0525	0.0221	0.1182	****	0.9627	0.9545	0.9544	0.9648	0.9655	0.9391	0.9573
BR	0.0456	**0.0188**	0.1042	0.0380	****	0.9784	0.9648	0.9734	0.9733	0.9466	0.9650
AJRJ	0.0464	0.0379	0.0981	0.0466	0.0218	****	0.9678	0.9669	0.9640	0.9362	0.9460
KRJ	0.0570	0.0345	0.1196	0.0467	0.0358	0.0328	****	0.9800	0.9630	0.9351	0.9463
JUP	0.0547	0.0209	0.1265	0.0358	0.0269	0.0337	0.0202	****	0.9722	0.9440	0.9637
JDRJ	0.0547	0.0232	0.1185	0.0352	0.0270	0.0366	0.0377	0.0282	****	0.9525	0.9563
BK	0.0589	0.0524	0.1069	0.0628	0.0548	0.0659	0.0671	0.0282	0.0487	****	0.9311
AJG	0.0681	0.0278	**0.1502**	0.0436	0.0357	0.0555	0.0552	0.0369	0.0447	0.0713	****

BP: Bhatinda, Punjab; BR: Barmer, Rajasthan; JDRJ: JNVU - Jodhpur, Rajasthan; AJRJ: AFRI - Jodhpur, Rajasthan; KRJ: Kota, Rajasthan; JG: Junagarh, Gujarat; DG: Diyodar, Gujarat; AJG: Agri - Junagarh, Gujarat; KMP: Khandwa, Madhya Pradesh; JUP: Jhansi, Uttar Pradesh; BK: Belgaum, Karnataka

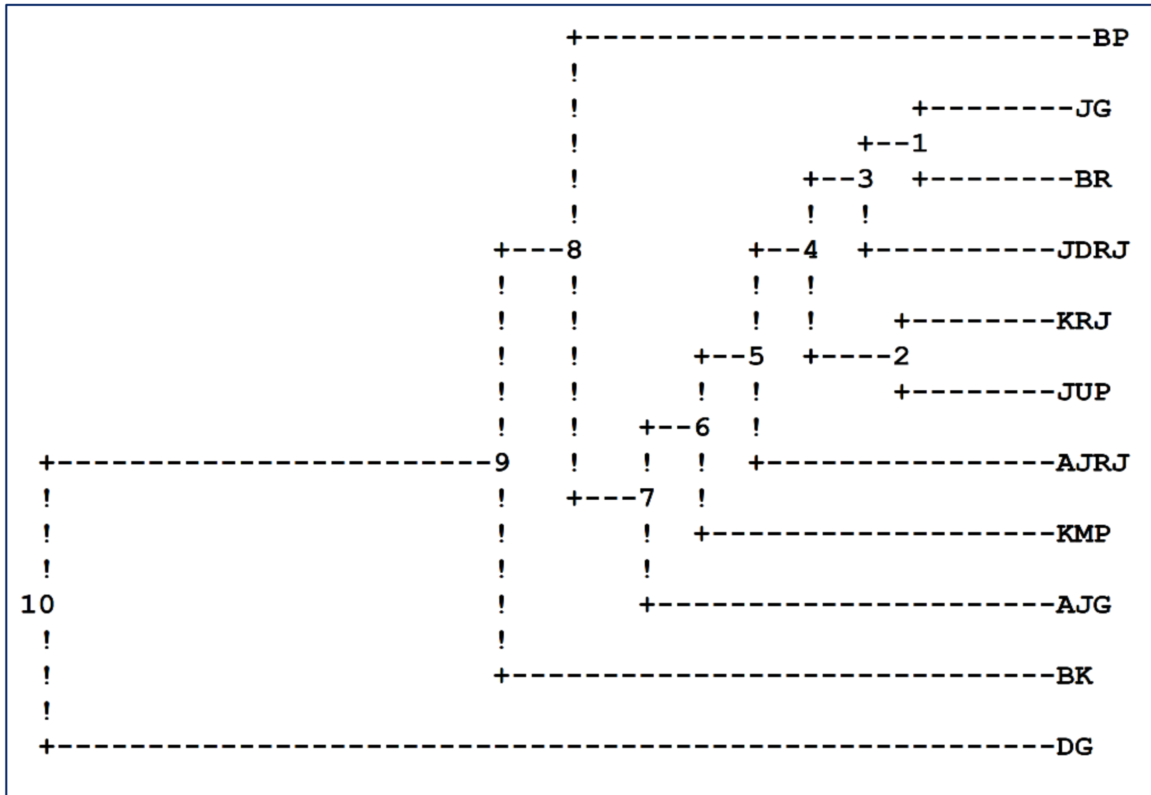


Figure 12: UPGMA based dendrogram of Nei' s genetic identity of location wise *Leptadenia reticulata* samples (see Table 1 for location code).

4.1.4. Principal coordinates analysis (PCoA)

The PCoA analysis provided a graphical representation of the genetic relationships between different populations of *L. reticulata* by defining the amount of variation captured by the first three axes (Figures 13 and 14). The primary axis was responsible for 5.73% of this variation, followed by 4.86% for the second axis, and 4.51% for the third. The bi-dimensional PCoA plot demonstrated that the principal coordinates one and two contributed to 5.73% and 4.86% of the overall genetic diversity, respectively. Analysis of the clusters within this plot highlighted distinct distributions for each of the eleven *L. reticulata* populations. The PCoA bi-plots revealed a scattered or intermingled pattern in the distribution of *L. reticulata* accessions, which was not strictly correlated

with their geographical origins (as illustrated in Figure 13). In the RAPD-based PCoA, the variation percentages were different: 9.44% for the first axis, 6.83% for the second, and 5.79% for the third (as seen in Figure 14). In this RAPD-based analysis, the BP and DG populations were markedly dispersed compared to others, whereas AJRJ, BK, and BR populations were more intermingled. The rest, including JG, KMP, KRJ, JUP, JDRJ, and AJG populations, exhibited overlapping patterns (as indicated in Fig. 14).

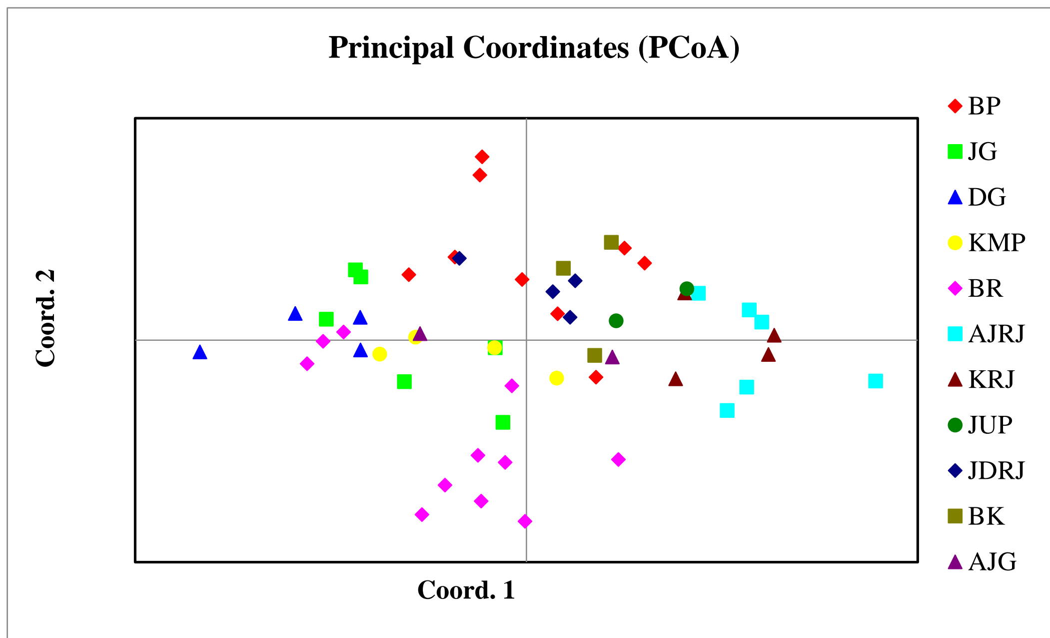


Figure 13: ISSR-based Principal Coordinate Analysis showing clustering of individual samples belonging to 11 locations of *Leptadenia reticulata* (see Table 1 for code location).

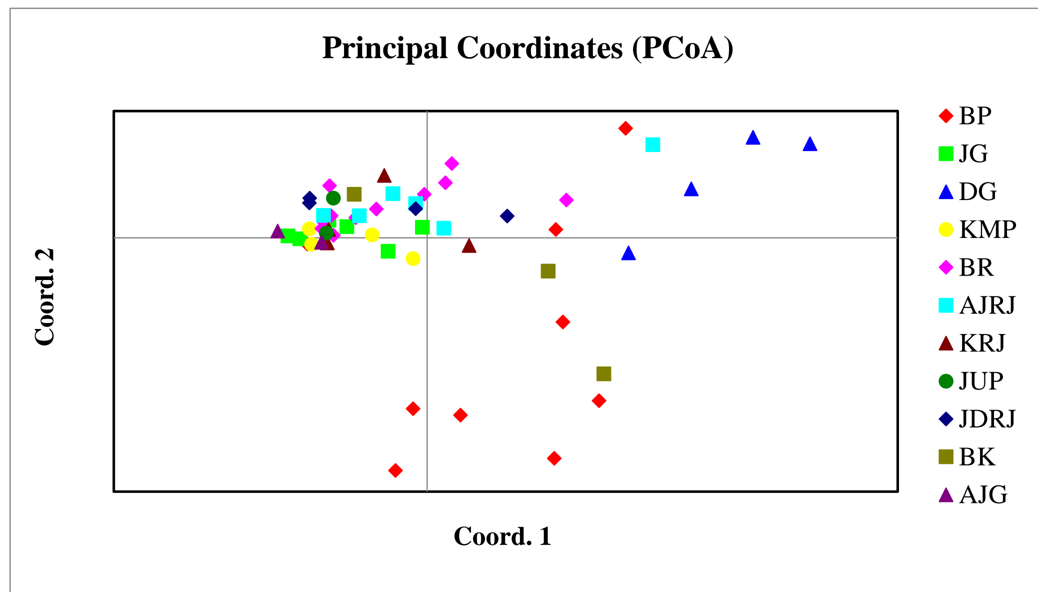


Figure 14: RAPD-based Principal Coordinate Analysis showing clustering of individual samples belonging to 11 locations of *Leptadenia reticulata* (see Table 1 for code location).

4.1.5. Principal component analysis (PCA)

PCA analysis was performed to know the connection and pattern of distribution using binary matrix from the genetic analysis (Figure 15 and 16). The variance proportion produced by binary matrix dataset including both ISSR and RAPD. ISSR dataset utilizing 287 scored loci within the study revealed proportion of variance ranging from 0.05035 to 0.00629 (Figure 15) and for that RAPD, 0.08438 to 0.00124 using 135 loci in the study (Figure 16). All accessions revealed a dispersed (ISSR) or intermixed (RAPD) distribution of *L. reticulata* accessions in PCA analysis.

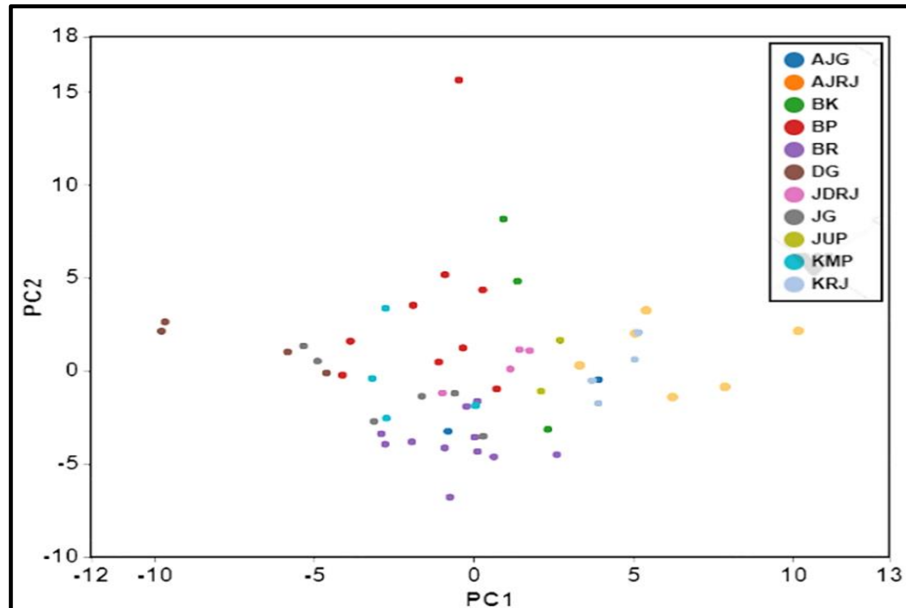


Figure 15: ISSR-based Principal Component Analysis showing clustering of individual samples belonging to 11 locations of *Leptadenia reticulata* (see Table 1 for code location).

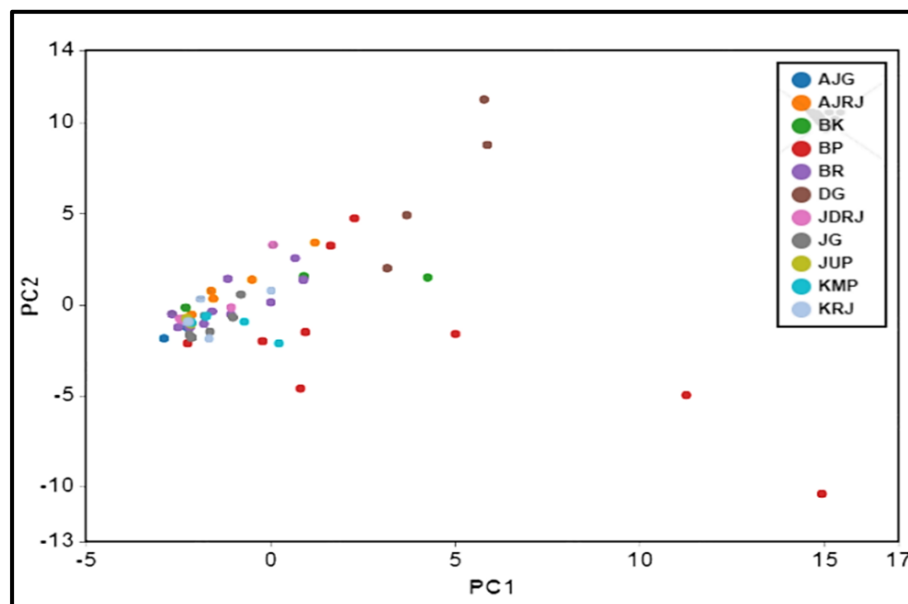


Figure 16: RAPD-based Principal Component Analysis showing clustering of individual samples belonging to 11 locations of *Leptadenia reticulata* (see Table 1 for code location).

4.2. To investigate the phytoconstituent variation in *Leptadenia reticulata* using fingerprinting method

4.2.1. Development and Validation of the Method

Upon examination under UV illumination, it was discerned that 300 nm was the most suitable wavelength for obtaining the spectrum. The selection of the mobile phase was the result of extensive experimentation, ultimately settling on a blend of toluene, chloroform, ethyl acetate, and formic acid in a proportion of 2.5:2:4.5:1 (v/v/v/v). This specific combination proved effective in achieving distinct separation in both the standard and the test sample, with the spectral results illustrated in Figure 17. Subsequently, the HPTLC method that was formulated underwent validation for various parameters at the 300 nm wavelength, as depicted in Figure 18.

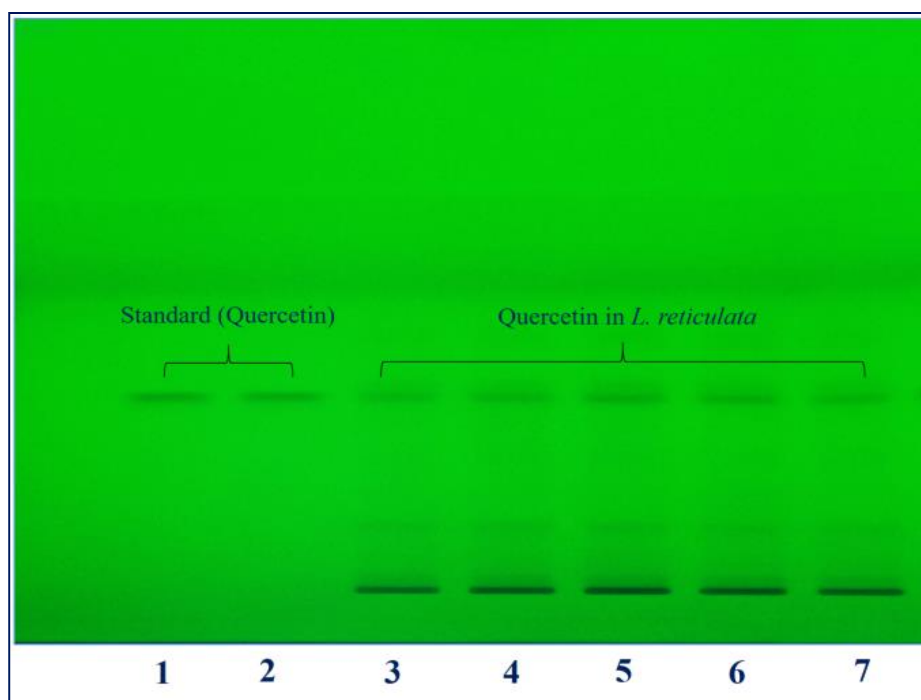


Figure 17: Developed HPTLC plate. Lanes 1-2: Standard (Quercetin); Lanes 3-7: *L. reticulata* samples (HT, DG, BK, BP and BR).

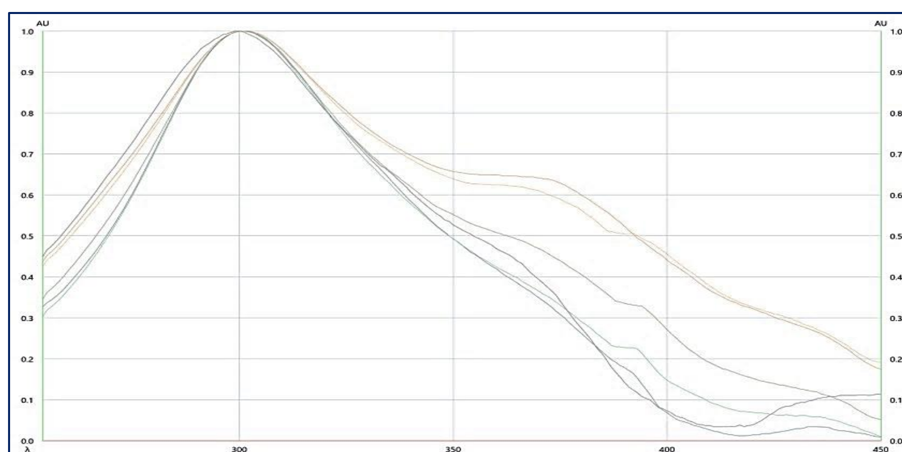


Figure 18: Absorption spectra of quercetin and plant samples of *L. reticulata*.

4.2.1.1. Calibration Curve

The method's linearity was confirmed over a range of 60-160 ng/band. This was evidenced by a correlation coefficient of 0.993 and a variation coefficient standing at 0.40%. The linear regression data are detailed in Table 16, while the associated calibration curve is represented in Figure 19.

Table 16: Analytical method validation parameters for quercetin (n = 6).

Parameters	Results
Linearity range (ng/spot)	60 -160
R_f	0.49
R^2	0.993
Regression equation	y = 0.0014x + 0.0002
Slope	0.001375
Intercept	0.000171
LOD (ng/spot)	0.40
LOQ (ng/spot)	32.9

R_f: Retention factor; R^2: Correlation coefficient; y: peak area; x: concentration (ng/band); LOD: limit of detection; LOQ: limit of quantification.

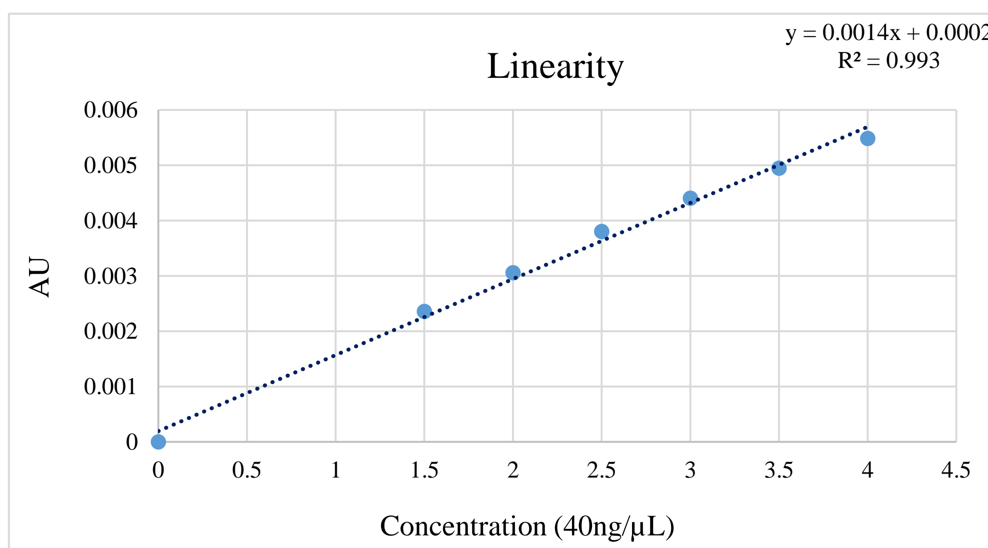


Figure 19: Calibration curve for quercetin.

4.2.1.2. Determining LOD and LOQ

Using the method outlined above, the Limit of Detection (LOD) and Limit of Quantification (LOQ) were ascertained to be 0.40 ng/band and 32.9 ng/band, respectively. These findings demonstrate that the method we developed offers superior sensitivity compared to existing techniques, as evidenced by its lower LOD and LOQ values.

4.2.1.3. Precision

The study focused on evaluating the repeatability, intermediate precision, and accuracy by utilizing the coefficient of variation (CV, %). This evaluation was conducted at different quercetin concentrations, specifically 80, 100, and 120 ng/spot, as outlined in Table 17. The precision within the same day (intra-day) and across different days (inter-day) was determined to be 1.63% and 1.87%, respectively. To verify the method's repeatability, a set of six quercetin samples at an identical concentration was

examined following the same experiment parameters. The method's precision and reproducibility were confirmed by the results, which were within an acceptable range. These findings are comprehensively presented in Table 17.

Table 17: Compiled data on intra-day and inter-day variability for quercetin analysis.

Standard marker	Concentration (ng)	Intra-day		Inter-day	
		% RSD	Mean RSD	% RSD	Mean RSD
Quercetin	80	1.87	1.63	1.98	1.87
	100	1.59		1.84	
	120	1.44		1.79	

% RSD: Percentage of relative standard deviation

4.2.1.4. Recovery

In evaluating the recovery of quercetin, the method involved spiking with additional quantities of 80%, 100%, and 120%. The recovery rate for quercetin was found to range between 95% and 105%, as detailed in Table 18. The relative standard deviation (RSD) for quercetin recovery varied from 0.75% to 2.5%, indicating a reliable recovery rate (see Table 18).

Table 18: Recovery data of quercetin and sample.

Sample area	Standard area (%)	Spiked area	Recovery %	Average %	RSD %
0.00143	0.00034 (80)	0.00187	105	101.70	1.3
	0.00100 (100)	0.00254	104		0.75
	0.00194 (120)	0.00322	95		2.5

% RSD: Percentage of relative standard deviation

4.2.1.5. Specificity

The method's specificity has been determined by comparing the R_f value (0.49) and the position of quercetin in both the standard and the sample. This comparison revealed a match, affirming the method's specificity. Chromatograms illustrating this comparison for both the standard and the sample are presented in Figure 20.

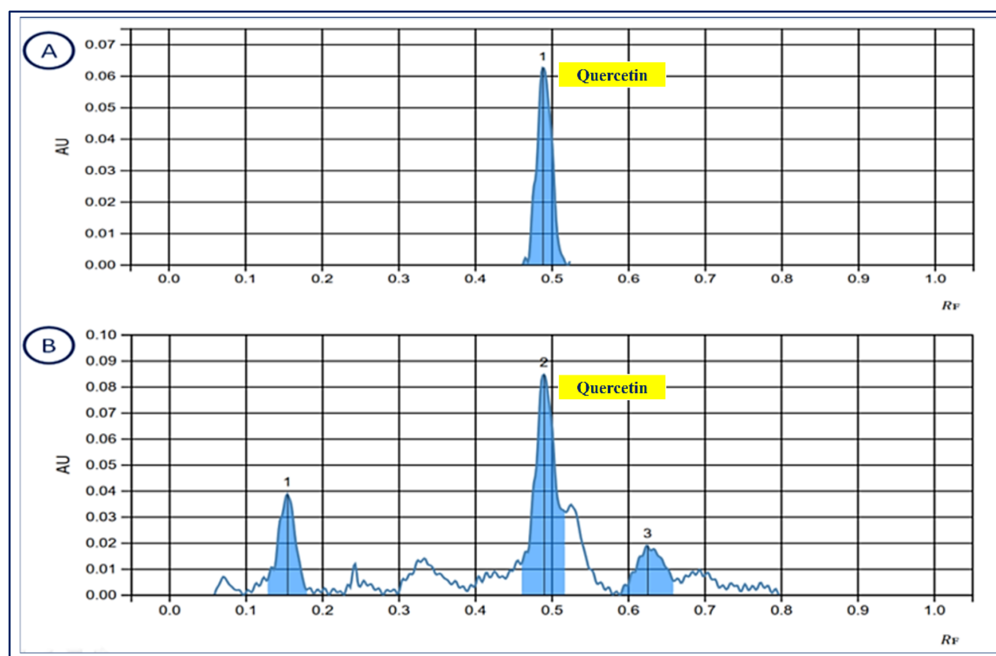


Figure 20: HPTLC Chromatograms of (A) Standard (quercetin) and (B) leaf extract of *Leptadenia reticulata*.

4.2.1.6. Robustness

The robustness of the method was assessed by observing the standard deviation of peak areas for each parameter. The RSD values obtained were within the acceptable range, demonstrating the method's robustness. This was further evidenced by the minimal RSD percentages observed following minor deliberate modifications to the established HPTLC method (as shown in Table 19).

Table 19: Robustness method (n = 3) for quercetin.

Quercetin	Mobile phase composition	
Amount (ng/spot)	toluene: chloroform: ethyl acetate: formic Acid (2.5:2:4.5:1) % RSD	toluene: chloroform: ethyl acetate: formic Acid (2:2:5:1) % RSD
100	1.59	1.92
120	1.44	1.01

% RSD: Percentage of relative standard deviation

4.2.2. Quantification of Quercetin in *L. reticulata*

Using the validated method, the quercetin content in *L. reticulata* leaves was quantified. The range of quercetin content found in five different *L. reticulata* accessions from various regions of India was between 6.73 and 13.84 mg/g. Notably, the highest quercetin content (13.84 mg/g) was detected in the BR (Bathinda, Punjab) region, while the lowest (6.73 mg/g) was in the HT (Hyderabad, Telangana) region (details provided in Table 20 and Figure 21).

Table 20: Content of quercetin in *L. reticulata* from geographical regions.

S. No	Accession No	Geographical Cultivation Region	Quercetin Content (mg/g)
1.	HT	Hyderabad (Telangana)	06.73 ± 0.020
2.	DG	Diyodar (Gujarat)	10.62 ± 0.036
3.	BK	Belagavi (Karnataka)	07.44 ± 0.035
4.	BP	Bathinda (Punjab)	13.84 ± 0.032
5.	BR	Baran (Rajasthan)	11.03 ± 0.021

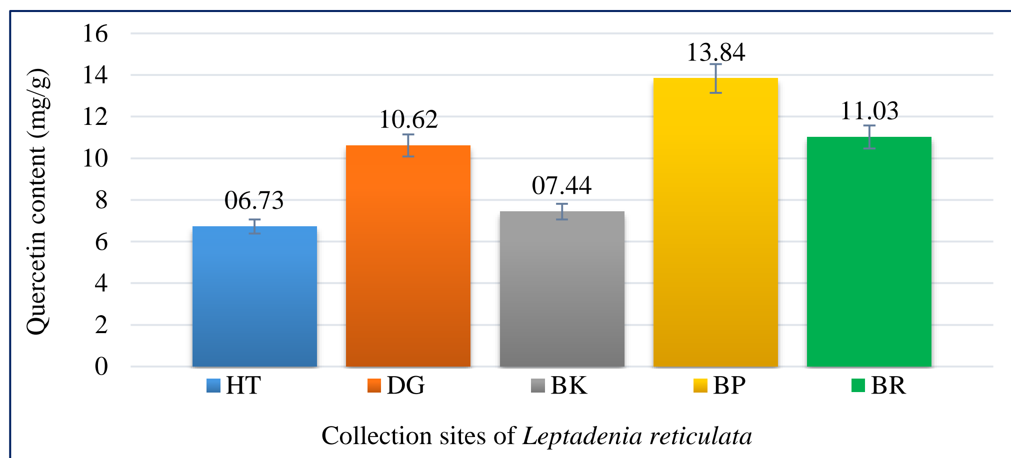


Figure 21: Variation of quercetin compound in *Leptadenia reticulata* samples.

Here, HT: Hyderabad, Telangana; DG: Diyodar, Gujarat; BK: Belagavi, Karnataka; BP: Bathinda, Punjab; BR: Baran, Rajasthan

4.3. To develop the molecular markers for identification and differentiation of *Leptadenia reticulata* from its adulterants/substitutes

4.3.1. Assay using ITS2 marker based DNA bar-coding

The ITS2 region of *L. reticulata* and its adulterants was amplified and verified using 1.5% agarose gel, as depicted in Figure 22. Sequences obtained from the ITS2 region of *L. reticulata* and adulterants (*W. volublis* and *H. ada-kodien*) were mined and aligned using online tools like MEGA and BioEdit. The National Centre for Biotechnology Information-Basic Local Alignment Search Tool (NCBI-BLAST) was used to compare these sequences with existing sequences, as shown in Figure 23. The alignment showed over 90% identity with NCBI-BLAST. A maximum likelihood-based dendrogram was constructed from the retrieved samples, forming two major groups: Group I containing all *L. reticulata* samples and Group II consisting of its adulterants (*W. volublis* and *H. ada-kodien*), as illustrated in Figure 24.

4.3.2. HRM analysis

Selective amplification of a genetic locus corresponding to ITS2 barcode region of *L. reticulata*, distinctly different from its adulterants which was achieved using primer specifically designed in this study. The performance of this primer pair to successfully differentiate from *L. reticulata* its adulterants was found to be the best from among the various primer pairs designed. Investigation of the HRM based curves with ITS2, is represented in Figure 25 where most of the *L. reticulata* samples showed unique profiles that could be easily distinguished. Furthermore, evaluation difference in melting curve of *L. reticulata* resulted in generation of unique profile close to each other (Figure 25).

4.3.3. Conventional PCR

Additionally, conventional PCR was performed from the primer pair successfully employed for HRM analysis. A consistent single 172bp band was obtained in *L. reticulata* (Figure 26) and there were no visible bands in adulterants. Bar-HRM analysis was found to be potentially useful and highly sensitive method to discriminate *L. reticulata* from adulterants

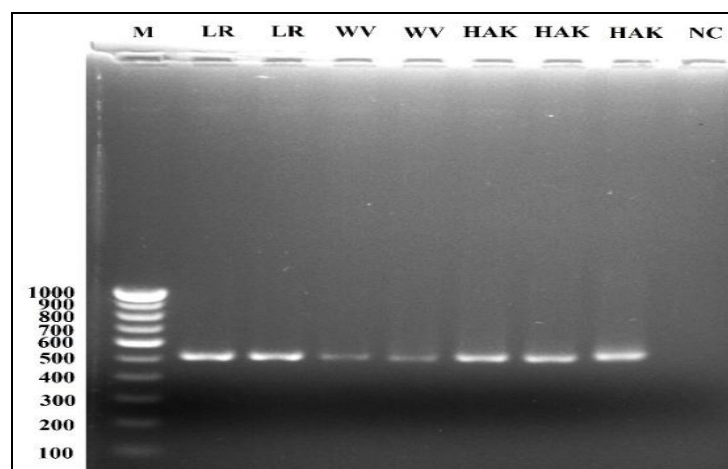


Figure 22: Agarose gel profile of PCR product of the ITS2 region and its amplicon size, 550bp.
(Here the code samples, LR: *L. reticulata;* WV: *W. volubilis* and HAK: *H. ada-kodien*).

Figure 23: Markers identified form the sequenced samples.

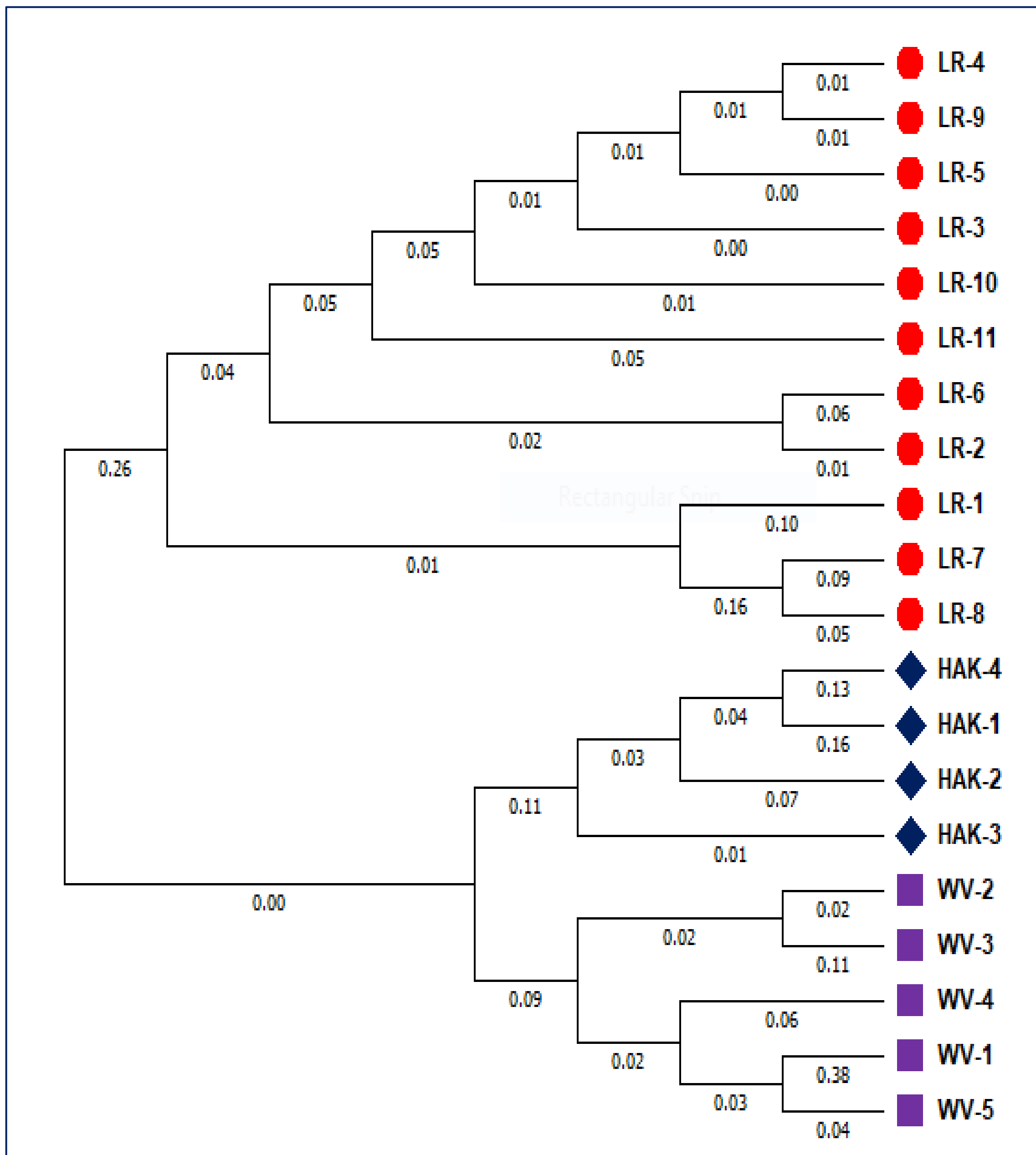


Figure 24: ITS2-derived molecular phylogenetic analysis of the *L. reticulata*, *W. volubilis* and *H. ada-kodien* samples.

(Here the samples code, LR: *Leptadenia reticulata*; WV: *Wattakaka volubilis* and HAK: *Holostemma ada-kodien*)

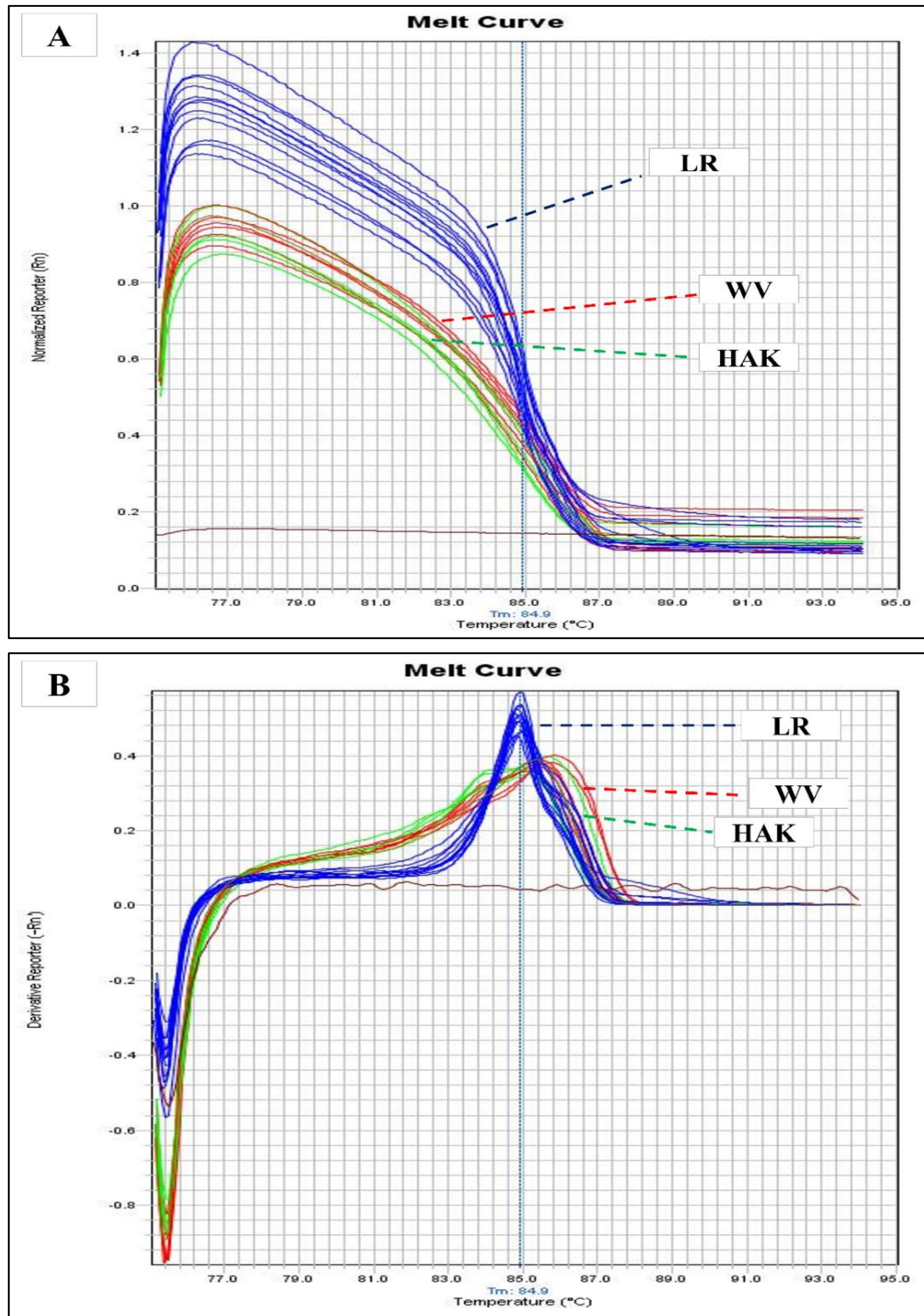


Figure 25: Differential plot for *L. reticulata* and its adulterants.

Here: Blue peaks: *L. reticulata*, Red and green lines: adulterants (*W. volubilis* and *H. ada-kodien*) and Brown line: control.

(Samples code- LR: *L. reticulata*; WV: *W. volubilis*; HAK: *H. ada-kodien*)

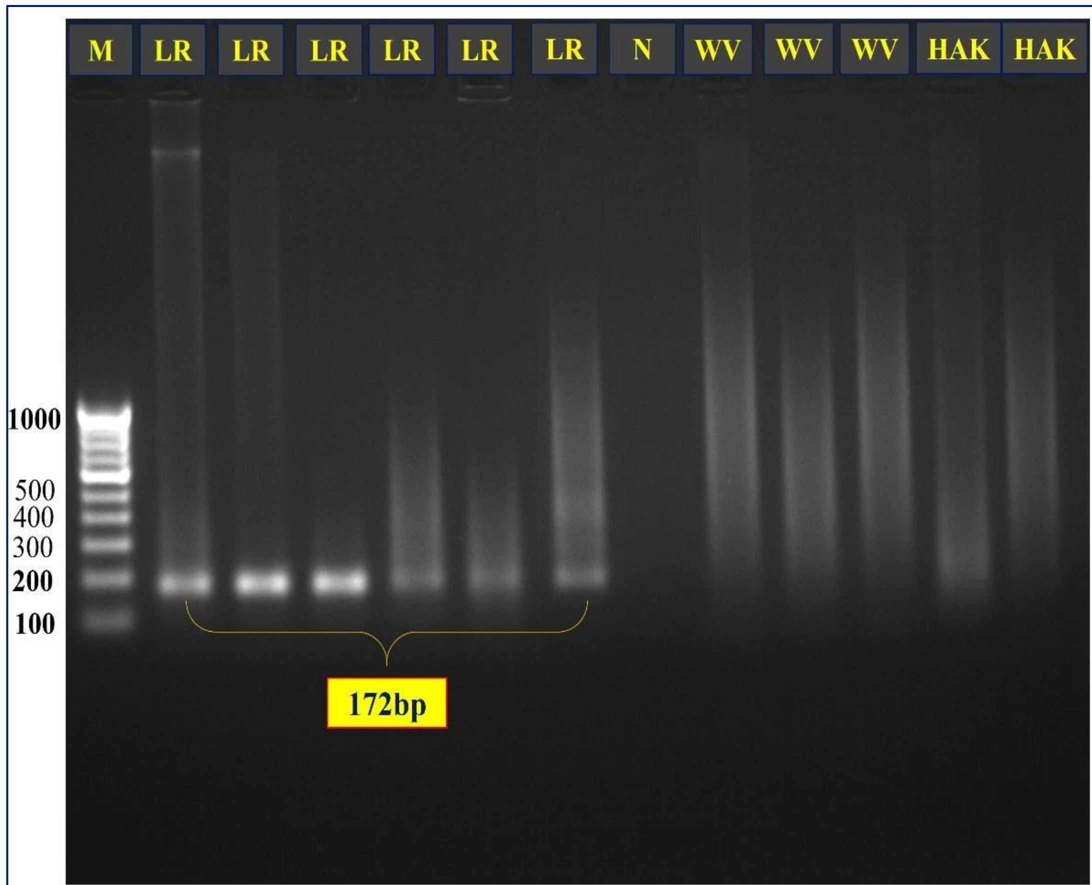


Figure 26: The result of agarose gel profile.

Here: Agarose gel profile of PCR and its amplicon size (172bp). [M: DNA ladder (100bp); LR: *L. reticulata*; WV: *W. volubilis*; HAK: *H. ada-kodien*; and NC: Negative control].

Chapter- 5
Discussion

5. Discussion

Molecular markers have become invaluable in identifying genetic variations in plants, enhancing our understanding of their distribution patterns.[40, 128] Recent advancements in genetic marker technologies enable the detection of such variations.[129, 130] Marker-based techniques are widely used in plant studies, though some types of molecular markers have inherent limitations.[129] When assessing intra and inter population diversity in plant species, it is typically advised to use a combination of several marker types.[131] Assessing genetic variations of the preserved germplasms by RAPD and ISSR techniques is easy and simple methods wherethe amplification happens across the total genome utilizing random primers.[40, 129-132]

For chemical constituent quantification, HPTLC stands out as a robust, straightforward, rapid, reliable, and effective analytical method. It excels in detecting, separating, and analysing a wide array of phytochemicals present in herbal extracts, offering superior analytical precision and accuracy in a short time and at a reasonable cost.[133-135] However, genotypic information alone is not always conclusive. Therefore, examining the extent of variations in phytochemical constituents in relation to genetic types can provide valuable insights for improving conservation methods and the proper utilization of resources.[35, 45] Analysing chemical diversity is crucial for understanding the composition of secondary metabolites and developing quality control parameters.[34]

5.1. Polymorphism

In this study, 55 samples of *L. reticulata*, collected from 11 different locations, were analysed using ISSR and RAPD assays. These techniques revealed unique banding patterns, highlighting the genetic diversity among the samples. Specifically, 9 ISSR and 5 RAPD markers produced banding patterns corresponding to 287 and 135 genetic loci,

respectively, demonstrating a high degree of polymorphism. Previous studies involving 15 samples of *Leptadenia pyrotechnica* showed lower polymorphism rates (41.7% and 39.5%) when using these markers.[114] The polymorphism level (ISSR: 84.61% and RAPD: 85.36%) observed in *Gymnema sylvestre* plant that belongs to Apocyanaceae.[136] Notably, higher polymorphism levels were observed in other plant species such as *Rosa damascene* and *Vitis vinifera*.[137, 138] PIC was used in our investigation as one of the genetic diversity metrics. The average PIC range, 0.283 in ISSR and 0.191 in RAPD revealed polymorphism level is medium. In general, ranges of PIC like PIC > 0.5 is considered to be high, 0.5 >PIC > 0.25 is medium and also for PIC < 0.25 is considered a low level of polymorphism.[139] The observed high polymorphism in this study could be attributed to the extensive geographical range from which *L. reticulata* samples were collected, as well as the effectiveness of the primer sets used.

5.2. Genetic Diversity

Analysis of molecular variance (AMOVA) indicated that only a small fraction of genetic variance in *L. reticulata*, 11%, was attributable to differences among populations, as determined by ISSR markers. In contrast, a significant 89% of the variance was found within populations. RAPD markers showed a similar trend, with only 3% of variance among populations and 97% within them. This pattern of molecular variance within *L. reticulata* populations (89%) was more pronounced than that observed in *L. pyrotechnica* (73%).[114] A comparable pattern of genetic variation was noted in *Madhuca indica*, with 85% of the variance within and 15% among its eight populations.[140] The AMOVA detected using RAPD markers showed genetic variation 98% within 2% in 32 diverse Malus genotypes.[141]

Investigating population genetic characteristics, such as Nei's genetic diversity (*h*) and Shannon's Information Index (*I*), showed comparable fluctuations in the eleven *L. reticulata* populations when analysed using ISSR and RAPD techniques. The Nei's gene diversity index between the populations showed lower diversity (ISSR: 0.00 - 0.23 and RAPD: 0.00 - 0.24). The similar results was observed in *Gymnema sylvestre* (ISSR: 0.00 - 0.20 and RAPD: 0.00 - 0.30).[136] The observed effective allele numbers were substantial (ISSR: 1.397 ± 0.366; RAPD: 1.398 ± 0.316), pointing to a relatively high genetic diversity within the *L. reticulata* groups. This diversity might be attributed to the diverse geographical locations from where the samples were collected and/or the types of markers employed.[41] Furthermore, variations between species also played a role in the observed high polymorphism levels. Several elements, such as reproductive strategies, population scale, historical population reductions, genetic drift, and gene migration, are known to affect genetic diversity in populations.[76, 142]

The genetic differentiation in Nei's model (G_{st}), which is measured on a scale from 0 to 1, with higher figures indicating more variation among populations, was recorded as 0.322 (ISSR) and 0.324 (RAPD) for *L. reticulata*. This suggests that the genetic differentiation between its populations is not very high. Genetic differentiation (ISSR: 0.41 and RAPD: 0.35) was recorded in *Gymnema sylvestre* which was high compare to *L. reticulata.*[136]

However, the estimated gene flow (Nm) values of 1.05 (ISSR) and 1.04 (RAPD) indicate modest gene exchange among populations, a characteristic often seen in rare and endangered species.[120] Gene flow (ISSR: 0.71 and RAPD: 0.92) was observed between the locations of *Gymnema sylvestre* which was low compare to *L. reticulata*.[136] Gene flow in natural populations is categorized as minimal ($Nm < 1$), moderate ($Nm > 1$), or

extensive (Nm > 4).[144, 145] It is recognized that enhanced genetic diversity, which facilitates gene flow, is often linked to better adaptation.[146, 147]

Factors such as habitat fragmentation, limited seed dispersal, and ecological impacts, including commercial exploitation of seeds, flowers, and bark, can lead to low gene flow.[51, 148, 149] Human activities, along with geographical barriers between populations, may also restrict gene flow in *L. reticulata*.

To understand the relationships among *L. reticulata* populations, ISSR and RAPD datasets were analysed using Nei's genetic distance-based UPGMA dendrogram. In the ISSR analysis, the populations formed two major clusters: Cluster I included 10 populations (population codes: KRJ, AJRJ, JG, BR, KMP, BP, JUP, JDRJ, AJG, and BK), while Cluster II comprised a single population (population code: DG). The genetic distance among these populations ranged from 0.035 (AJRJ-KRJ) to 0.164 (JUP-DG). A location-based dendrogram showed that the DG location was genetically distinct, while the others were intermixed, as indicated in the accession-based cluster analysis.

On the other hand, two major clusters produced by RAPD based analysis, which is similar to ISSR based analysis. The genetic distances between BR-JG and AJG-DG were 0.018 and 0.150, respectively. The UPGMA dendrogram based on Nei's genetic distances highlighted the highest and lowest similarities between BR and JG, and AJG and DG, respectively. However, the geographical locations of the sample collections, with a few exceptions, were generally consistent with their relative geographical positions. The observed deviations in the cluster analysis could be attributed to variations in allele frequencies within the gene pool, influenced by gene flow.[38]

The Principal Coordinate Analysis (PCoA) of ISSR and RAPD markers revealed distinct clustering patterns. In the ISSR analysis, the graphical representation indicated unique distributions for each of the eleven *L. reticulata* populations. This analysis showed a varied or intermingled arrangement of *L. reticulata* accessions within the PCoA bi-plots. Conversely, the RAPD-based PCoA exhibited a pronounced scattering of the BP and DG populations, distinct from others. In contrast, the AJRJ, BK, and BR populations demonstrated a mixed pattern. The remaining populations, JG, KMP, KRJ, JUP, JDRJ, and AJG, showed overlapping characteristics. In previous study, all the population was shown in scattered areas using PCoA for same genus of Leptadenia.[114]

5.3. Phytochemical Analysis

An analysis of the plant's phytoconstituents identified a variety of flavonoids, glycosides, and phenolic substances. Bioactive components that have been recognized in *L. reticulata* include rutin, quercetin, apigenin, α-amyrin, β-sitosterol, stigmasterol, β-amyrin, ferulic acid, diosmetin, hentriacontanol, simiarenol, and many alkaloids. These elements are recognized for their notable pharmacological effects.[11] Notably, *L. reticulata* does not possess a unique, singular phytochemical marker. This study aims to improve our understanding of the phytochemical diversity of *L. reticulata* and to evaluate the impact of differing environmental conditions.

5.4. Compound Content variation in *L. reticulata*

The quercetin content in the leaf extract of *L. reticulata* samples exhibited approximately a 99% variance between the highest and lowest levels. This variation is thought to be influenced by factors such as the plant's developmental stage, environmental conditions, and genetic makeup.[150] In addition, other aspects like extraction methodologies adopted may also influence the particular content of the

metabolites present.[151, 152] Thus, there is evidence that consuming flavonoids may reduce the chance of developing cancer and heart disease.[12, 13]

Quercetin, in particular, is utilized in treating various conditions such as cancer, cardiovascular diseases, allergies, diabetes, and possesses immunomodulatory and anti-infective properties.[14-15] Although quercetin content in *L. reticulata* has been detected using HPTLC and HPLC methods, aspects like linearity, precision, and recovery were not previously established.[11, 27] This study developed and validated a method for quantifying quercetin in *L. reticulata*, capable of detecting even minimal quercetin levels. The quercetin content ranged from 6.73 ± 0.020 to 13.84 ± 0.032 mg/g in five *L. reticulata* accessions collected from different regions of India. The highest quercetin level (13.84 ± 0.032 mg/g) was found in the BP sample (from Bathinda, Punjab), while the lowest (6.73 ± 0.020 mg/g) was in the HT sample (from Hyderabad, Telangana). Research on *Gloriosa superba* from various districts in India revealed notable differences in the levels of colchicine.[152] Additionally, an investigation was conducted to assess the variability in curcumin levels in *Curcuma longa* L (turmeric) sourced from eight distinct Indian regions. This study found the highest concentration of curcumin in turmeric from Erode (Tamil Nadu), while the lowest was in samples from Surat (Gujarat). This suggests that the turmeric variety from Erode (Tamil Nadu) is of superior quality compared to other regions in India.[84] A similar examination was carried out to measure the chrysin content in *Oroxylum indicum* vent, collected from various parts of the plant in different Indian regions and observed that there was a significant difference in chrysin levels between plants from the Western Ghats and those from U.P.[85] The variations in phytoconstituent levels noted in these studies could be attributed to geographical and seasonal factors, as these elements significantly influence the composition of plant species.[150]

5.5. Assay Using ITS2 Barcode

DNA barcoding, particularly using sequences from the ITS region, is becoming a prevalent tool for taxonomic identification and differentiation of plant species.[153] The ITS2, a noncoding nuclear region, has proven to be a valuable sequence for plant DNA barcoding, especially in medicinal plants. The use of HRM analysis is emerging as a straightforward, effective, and robust technique to detection of plant species.[154]

Given the increasing market demand for *L. reticulata*, instances of adulteration with *W. volubilis* and *H. ada-kodien* are frequently reported. This study introduces a rapid, reliable, and sensitive assay for authenticating *L. reticulata* using HRM analysis, which includes specific oligonucleotides targeting the ITS2 region. In the phylogenetic analysis, *L. reticulata* samples formed a distinct group, separate from *W. volubilis* and *H. ada-kodien*, which were closely related and grouped together. Thus, *L. reticulata* and its adulterants were effectively differentiated using a maximum likelihood based dendrogram.

In this investigation, the Bar-HRM technique was employed to distinguish *L. reticulata* from its common adulterants (*W. volubilis* and *H. ada-kodien)*. The study involved amplifying template DNA using a specific primer set and PCR, followed by HRM analysis to estimate the Tm (melting temperature) values for each species, which were represented by distinct peaks.[154] This approach enabled the clear differentiation of two distinct DNA types using HRM analysis with one of the specially designed primer pairs. The melting curves for *L. reticulata* samples were notably different from those of *W. volubilis* and *H. ada-kodien*. The Bar-HRM analysis conducted in this research proved effective in detecting *L. reticulata* and distinguishing it from the other two species. Additionally, the Bar-HRM analysis supported the results of an ITS2 sequence-based

dendrogram, which indicated that commercial market samples lacked *L. reticulata* content.

HRM analysis is recognized for its sensitivity and practical advantages, such as being a closed-tube method that minimizes cross-contamination and eliminates the need for hazardous chemicals like acrylamide, formamide, and ethidium bromide.[155, 156] Discriminating species using universal primers is often challenging due to inconsistent results.[157] To the best of our understanding, this is the initial report of *L. reticulata* marker development employing real-time HRM analysis, which represents a substantial step in the development of a trustworthy technique for the selective identification of *L. reticulata* in crude drug samples. Furthermore, to establish a more cost-effective and straightforward identification method for *L. reticulata*, conventional PCR was conducted with the primer pair used in HRM analysis. The presence of 172 bp bands in *L. reticulata* samples and their absence in *W. volubilis* and *H. ada-kodien* samples suggest that conventional PCR is a viable method for detecting *L. reticulata* in raw powdered drug samples. Overall, the study demonstrated widespread substitution in commercial *L. reticulata* crude drug samples using ITS2 sequence-based HRM analysis. Bar-HRM analysis emerged as a highly sensitive and potentially effective method for differentiating *W. volubilis* and *H. ada-kodien* from *L. reticulata*.

Chapter- 6
Summary

6. Summary

The present study revealed that both the marker systems (RAPD and ISSR) showed 100% polymorphism among 55 *L. reticulata* accessions from eleven different regions of India. AMOVA indicated a high variance within populations (89% for ISSR and 97% for RAPD) and a lower variance among populations (11% for ISSR and 3% for RAPD). This high genetic variation suggests enhanced gene flow due to better adaptation. ISSR and RAPD markers indicated moderate gene flow and limited genetic differentiation among populations, possibly due to fragmentation patterns, low seed dispersal, and anthropogenic influences. Developed and validated HPTLC method was used to detect the quercetin content in geographically collected *L. reticulata* samples. Significant phytochemical variation was observed in *L. reticulata* samples, with the highest quercetin content found in the northern region compared to the southern region of India. Samples of common adulterants, *W. volubilis* and *H. ada-kodien*, were collected from various locations of India. To address these phytochemical variations, DNA profiling techniques, which are less affected by environmental factors, were employed. These techniques, including ISSR fingerprinting/DNA barcoding coupled with chemical fingerprinting, proved effective in detecting and differentiating *L. reticulata* from its common adulterants. Furthermore, the ITS2-based Bar-HRM analysis technique developed in this study has shown potential as an effective method for identifying and differentiating *L. reticulata* from *W. volubilis* and *H. ada-kodien*. The primer pairs derived from this study could also be applied in conventional PCR techniques to successfully distinguish *L. reticulata* from its common adulterants.

Chapter- 7
Conclusion

7. Conclusion

This research has endeavoured to elucidate both the genetic and phytochemical profiles of *L. reticulata*, a crucial and extensively traded raw drug source, which is currently experiencing rapid depletion, highlighting the urgent need for conservation efforts. The findings offer insights into the genetic diversity and variations in quercetin marker compounds, presenting a contemporary snapshot of the population genetic structure of *L. reticulata* across various regions of India. The study identified that the genotypes of *L. reticulata* predominantly group into two main clusters. These clusters exhibit a strong correlation with their geographic origins. However, the study noted significant disparities in the metabolites analysed, which did not align with the genotypic differences. The variations observed through AMOVA suggest a healthy degree of pollination in the natural environment, an encouraging sign ecologically.

The comprehensive analysis of population genetics conducted in this research provides valuable data for formulating conservation strategies tailored to this species. The observed moderate gene flow and limited genetic differentiation underscore the necessity of conserving the *L. reticulata* plant. Conservation tactics should incorporate multiple genotypes from diverse locations in reforestation and afforestation initiatives. Additionally, efforts should be made to reconnect fragmented populations through ex-situ plantations, fostering a merging of these populations. The variations in quercetin content obtained in *L. reticulata* from different regions, which is influenced by different factors, including climatic circumstances and other edaphic factors. The study's findings showed that climatic conditions may play an essential role in the characterization of phytochemicals in *L. reticulata*.

In addition, DNA barcoding methods were successfully demonstrated as a tool to

differentiate the adulterants of *L. reticulata* and ITS2 derived sensitive Bar-HRM analysis performed revealed detection of *L. reticulata* from its common adulterants like *W. volubilis* and *H. ada-kodien.* The technique can be used to even quantify the extent of genuine drug of *L. reticulata* present in a given sample. This study also provided the primer pairs, which could be used in conventional PCR to discriminate and differentiate *L. reticulata* from common adulterants like *W. volubilis* and *H. ada-kodien.*

Chapter- 8
Bibliography

8. Bibliography

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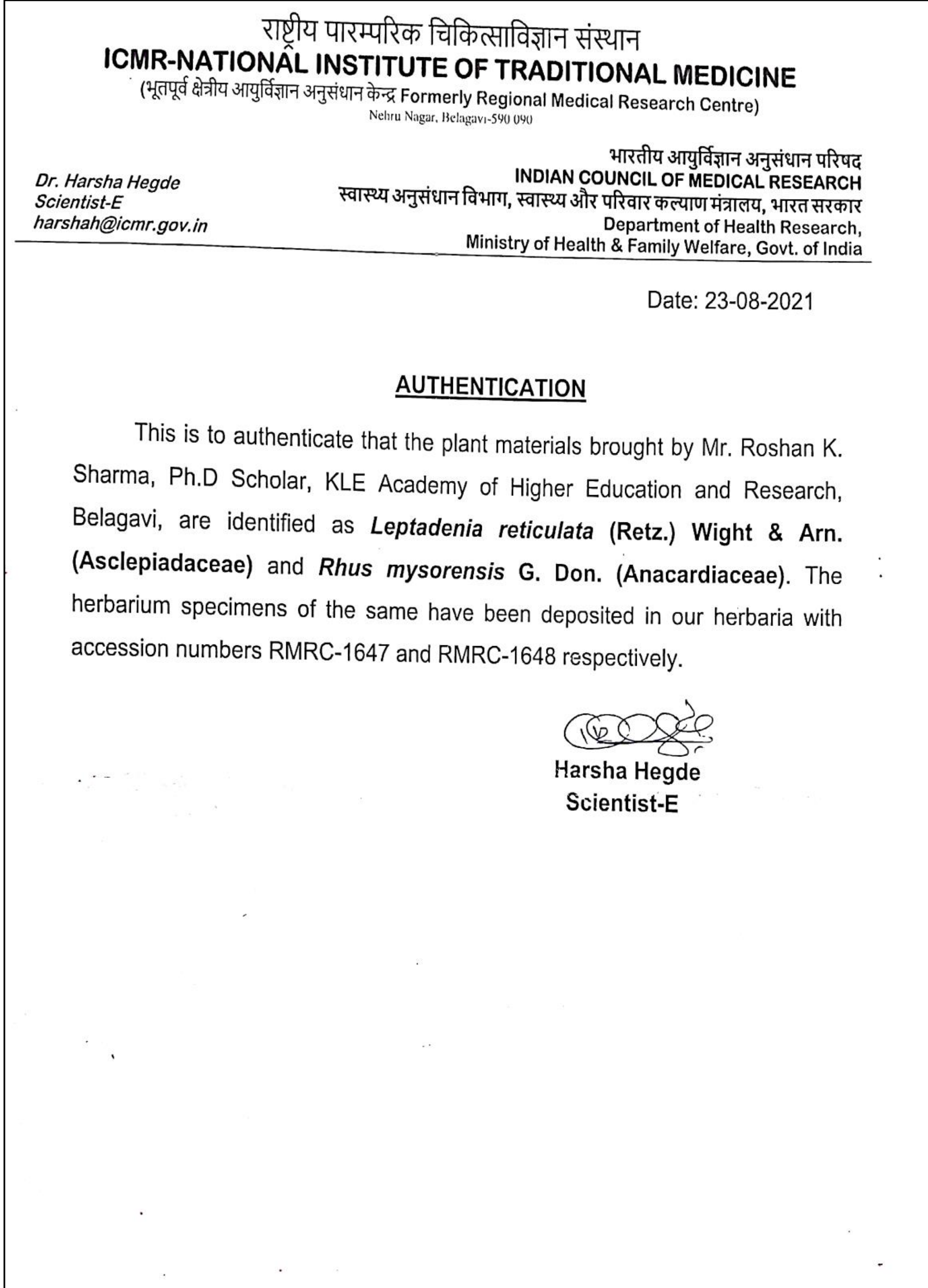
Chapter- 9
ANNEXURE

9. ANNEXURE

- **Plant authentication certificates**



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Date: 23-08-2021

AUTHENTICATION

This is to authenticate that the plant materials brought by Mr. Roshan K. Sharma, Ph.D Scholar, KLE Academy of Higher Education and Research, Belagavi, are identified as ***Leptadenia reticulata*** **(Retz.) Wight & Arn. (Asclepiadaceae)** and ***Rhus mysorensis*** **G. Don. (Anacardiaceae)**. The herbarium specimens of the same have been deposited in our herbaria with accession numbers RMRC-1647 and RMRC-1648 respectively.

Harsha Hegde
Scientist-E

PASSPORT DATA

Name of the drug (Sanskrit)	Hemajivanti
Name of the drug (Local)	Kodippalai
Botanical Name	*Dregea volubilis* (L.f.) Benth. ex Hook.f. syn. *Marsdenia volubilis* (L. f.) Cooke *Wattakaka volubilis* (L. f.) Stapf
Family	Apocynaceae
Part used/supplied	Leaves
Date of collection	25/03/2021
Place of collection	Anna Hospital Campus (Near Pharmacy Building), Chennai
Time of collection	11.00 a.m
Authenticated by	Dr. G. Kusuma, Research Officer (Ay.)/Scientist – 3, Captain Srinivasa Murthy Central Ayurveda Research Institute, Anna Hospital Campus, Arumbakkam, Chennai – 600106
Voucher Specimen Number	**CSMCARI/PS/ 0239**

PASSPORT DATA

Name of the drug (Sanskrit)	Arkapushpi, Jivanti
Name of the drug (Local)	Palay kira, Adapathiyan
Botanical Name	*Holostemma ada-kodien* Schult. syn. *Asclepias annularis* Roxb.
Family	Apocynaceae
Part used/supplied	Leaves (Sapling)
Date of collection	02/12/2020
Place of collection	Nursery of Arya Vaidya Pharmacy Factory at Kanjikode, Palakkad dist., Kerala.
Time of collection	3.30 pm
Authenticated by	Dr. G. Kusuma, Research Officer (Ay.)/Scientist – 3, Captain Srinivasa Murthy Regional Ayurveda Drug Development Institute, Anna Hospital Campus, Arumbakkam, Chennai – 600106
Voucher Specimen Number	**0237**

➢ Publications

1. **Sharma RK**, Jalalpure SS, Acharya R, Prasad BS, Hegde S. Molecular genetic diversity and biological activity assessment in *Leptadenia reticulata* from India. Gene Reports. 2023 Nov 29:101862.

2. **Sharma RK**, Jalalpure SS, Chouhan MK, Deshpande S, Acharya R, Hegde S. Decipher the inhibitory potential of phytocompounds from *Leptadenia reticulata* on dopamine D2 receptor to enhance prolactin secretion. Drug Research. 2022 Apr; 72 (04):189-96.

3. **Sharma RK**, Jalalpure SS, Acharya R, Prasad BS, Hegde S, Chauhan MK, Kurangi B, Patil U. Estimation of quercetin by high performance thin-layer chromatography and in vitro anti-inflammatory activity of *Leptadenia reticulata* (Wight & Arn.) from different parts of India. International Journal of Green Pharmacy (IJGP). 2023 Sep 15; 17 (03).

(*Front page of each article has been attached*)

➢ Presentations

1. **Sharma RK**, Jalalpure SS. "Quantification of variability in Quercetin content in *Leptadenia reticulata* collected from different geographical regions of India" 2023. National conference at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh (Second Prize: Best Oral Selected).

2. **Sharma RK**, Jalalpure SS, Acharya R, Prasad BS, Hegde S. "Authentication of the commercially important Ayurvedic medicinal plant ‘Jivanti’ using molecular methods" 2023. International conference at the National Institute of Ayurveda (NIA), Jaipur (Poster Presentation).

(*Certificates are attached*)

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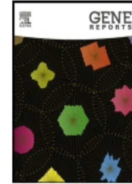

Molecular genetic diversity and biological activity assessment in *Leptadenia reticulata* from India

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ABSTRACT

Leptadenia reticulata (Retz.) Wight & Arn. (Jivanti) has enormous therapeutic value in traditional medicine because of its revitalizing, rejuvenating, and lactogenic properties. Due to overexploitation and a lack of habitat, the species is rapidly disappearing. Therefore, it is essential to understand the genetic diversity as well as the biological activity profile for conservation and its medicinal use. Nine ISSR markers were used to assess the genetic diversity of fifty-five *L. reticulata* samples that were collected from eleven locations in India for this study. ISSR markers yielded 287 bands overall and showed 100 % polymorphism. The genetic assessment showed that diversity was relatively high at the species level but lower at the population level. AMOVA revealed 89 % variation within and 11 % between *L. reticulata* locations. Limited genetic differentiation (G_{ST} = 0.32) and moderate gene flow (*Nm* = 1.05) were observed between the locations of *L. reticulata*. Simultaneously, the respective chemotypes of *L. reticulata* from eleven locations were assessed for their phenolic and flavonoid contents, as well as antioxidant, anti-inflammatory, anti-diabetic, and antimicrobial properties using aqueous extracts. Despite minor variations, the biological activities of *L. reticulata* from various locations in this study did not deviate significantly. The *in-vitro* activities determined support its efficacy in traditional medicine. The data from this study are valuable for the efficient management of germplasm for breeding and conservation purposes to meet the ever-increasing commercial demand for *L. reticulata* in the herbal drug industry.

1. Introduction

Leptadenia reticulata (Retz.) Wight & Arn. (Apocynaceae), usually familiar as 'Jivanti,' is a perennial, extremely branched, twining, and lactiferous climber found in the north and south regions of India and used to cure health-related disorders (Shekhawat et al., 2006; Nadkarni, 2007; Sudipta et al., 2014; Naik et al., 2018). One of the most significant medicinal herbs, *L. reticulata*, has a wide range of therapeutic applications that are documented in practically all classic Ayurvedic scriptures and is regarded as the best vegetable [ṣreṣṭhaśāka] (Naik et al., 2018). The utility of *L. reticulata* is increasing due to the numerous properties and commercial value of raw materials (INR 211/kg) on the market (Shekhawat et al., 2006). Currently, *L. reticulata* is listed as endangered by the Wildlife Institute of India under special habitats and threatened

Abbreviations: AMOVA, Analysis of Molecular Variance; CFU, Colony Forming Unit; DNSA, 3, 5 dinitro salicylic acid; dNTPs, Deoxynucleoside triphosphates; DPPH, 1, 1-diphenyl-2-picrylhydrazyl; EMR, Effective Multiplex Ratio; G_{ST}, Nei's genetic differentiation index among populations; *h*, Nei's Genetic Diversity; H_S, Genetic diversity within the population; H_T, Total Genetic Diversity; *I*, Shannon's Information Index; ISSR, Inter Simple Sequence Repeat; MBC, Minimum Bactericidal Concentration; MHA, Mueller Hinton Agar; MHB, Mueller Hinton Broth; MI, Marker Index; MIC, Minimum Inhibitory Concentration; *Na*, Observed number of alleles; NADH, Nicotinamide adenine dinucleotide; *Ne*, Effective number of alleles; *Nm*, Gene Flow; PIC, Polymorphism Information Content; PCoA, Principal Coordinate Analysis; PPB, Percentage of Polymorphic Band; REMA, Resazurin Microtitre Assay; SAHN, Sequential Agglomerative Hierarchical and Nested Clustering; TFC, Total Flavonoid Content; TPC, Total Phenolic Content; UPGMA, Unweighted Pair Group Method with Arithmetic Mean; UBC, University of British Columbia.

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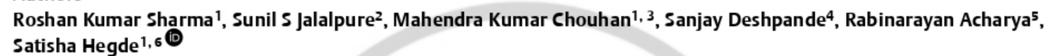

Decipher the inhibitory potential of phytocompounds from *Leptadenia reticulata* on dopamine D2 receptor to enhance prolactin secretion

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Key words

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ABSTRACT

Dopamine is secreted by the hypothalamus, which inhibits the proliferation and effectiveness of lactotroph cells that release prolactin via dopamine D2 receptor (D2R). D2R activation inhibits lactotroph cell prolactin synthesis and regulates prolactin gene expression. Although, commercial medications are available for hypogalactia and agalactia, various plant sources significantly alleviate these problems. *Leptadenia reticulata* (Jivanti) is one of the important medicinal plants often consumed by nursing mothers to improve breast milk production. However, mechanism and chemical constituents involved in the inhibition of D2R by Jivanti is unclear. Therefore, in this study the phytocompounds reported from Jivanti were used for in-silico analysis to predict D2R inhibitory potential. The binding affinity value of campesterol and β-sitosterol (−10.1 and −10.0 kcal/mol) with D2R has high revealed by molecular docking and stable interaction reveled by molecular dynamics simulation. Thus, these lead compounds could exert more D2R inhibitory activity resulting into prolactin release, which may lead to an increase in breast milk production. Although all selected compounds had fine permeation, non-toxic, and non-carcinogenic characteristics predicted by ADMET, campesterol had good solubility, absorption characteristics compared to other. Therefore, Jivanti, which is traditionally known medicinal plant, could be explored as a medication candidate to boost breast milk production.

ORIGINAL ARTICLE

Estimation of quercetin by high-performance thin-layer chromatography and *in vitro* anti-inflammatory activity of *Leptadenia reticulata* (Wight & Arn.) from different parts of India

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Abstract

Background: *Leptadenia reticulata* (Wight & Arn.) has made important contributions to both traditional and modern medicine. **Aims and Objectives:** The study aims to develop and validate an high-performance thin-layer chromatography (HPTLC) method for the determination of quercetin content in *L. reticulata* collected from various parts of the country and to assess its anti-inflammatory activity using the bovine serum albumin (BSA) inhibition method. **Materials and Methods:** Five samples were obtained from different geographical locations in India, and no significant variation was found in the morpho-anatomical study. HPTLC method developed using a toluene: chloroform: ethyl acetate: formic acid (2.5: 2: 4.5: 1) mobile phase at a wavelength of 300 nm. Second, anti-inflammatory activity of *L. reticulata* extract was assessed using the BSA inhibition approach. **Results:** For the standard and sample, R_f value of quercetin (0.49) was observed. Concentration range of quercetin (60–160 ng/spot) was determined to be linear for the linearity approach, with a regression coefficient for quercetin of 0.999 and an optimal recovery of 101.70%. The limits of detection and limits of quantification were found to be 0.78 and 2.36 ng, respectively. Study revealed quercetin content differed among *L. reticulata* collected from different geographical regions. The highest and lowest quercetin contents in *L. reticulata* were detected in LR-103 and LR-099, respectively. From the results obtained, sample LR-103 showed effective anti-inflammatory activity compared to LR-099 at certain concentrations. **Conclusion:** The developed method is suitable for the detection and quantification of the lowest content of quercetin in *L. reticulata*. The obtained data indicate that geographic origin and climatic conditions may be major factors in determining the phytocompounds and biological activities of *L. reticulata* plant species.

Key words: Anti-inflammation, high-performance thin layer chromatography, India, *Leptadenia reticulata*, Quercetin

INTRODUCTION

Herbal remedies have endured for a long time because of their safety, efficacy, folklore practice, and lack of adverse reactions. Herbal plants showed compatibility with human physiological mechanisms due to their natural properties. Most of the herbal plants have been employed in formulations as antidiabetic and antioxidant agents since ancient

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