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REVIEW ARTICLE

GENETIC MODIFICATION AND TRANSFORMATION STUDIES IN AROMATIC GRASSES: ADVANCES IN *CYMBOPOGON* AND *CHRYSOPOGON*

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Abstract: Aromatic grasses of the genera *Cymbopogon* and *Chrysopogon* (vetiver) represent economically important crops valued for their essential oils, phytoremediation capabilities, and diverse industrial applications. This manuscript provides a comprehensive review of genetic modification and transformation studies in these aromatic grasses, synthesizing recent advances in tissue culture, regeneration protocols, and genetic engineering approaches. The review examines in vitro regeneration systems, Agrobacterium-mediated transformation protocols, molecular characterization of essential oil biosynthesis pathways, and emerging biotechnological applications. Key findings reveal that while efficient regeneration systems have been established for multiple *Cymbopogon* species and vetiver, successful stable genetic transformation remains limited, with only a few reports of transgenic vetiver plants. The manuscript highlights critical bottlenecks in transformation efficiency, discusses the molecular basis of terpenoid biosynthesis, and identifies future directions for metabolic engineering and crop improvement in these valuable aromatic grasses.

Keywords: Aromatic grasses, *Chrysopogon zizanioides*, Genetic transformation, Essential oil biosynthesis

INTRODUCTION

Aromatic grasses belonging to the genera *Cymbopogon* and *Chrysopogon* represen t a diverse group of economically important plants cultivated worldwide for their essential oils, medicinal properties, and environmental applications. The genus *Cymbopogon*, comprising approximately 140 species, includes commercially significant species such as lemongrass (*C. citratus*, *C. flexuosus*), citronella (*C. winterianus*, *C. nardus*), palmarosa (*C. martinii*), and other aromatic grasses valued for their monoterpene-rich essential oils (Bhat *et al.*, 2023; Meena *et al.*, 2016). Vetiver (*Chrysopogon zizanioides*, syn. *Vetiveria zizanioides*), while taxonomically distinct, shares similar agronomic characteristics and has gained prominence for its sesquiterpene-rich essential oil and exceptional phytoremediation capabilities (Nakbanpote *et al.*, 2024; Obaleye *et al.*, 2023; Pandey and Praveen, 2020).

The economic importance of these aromatic grasses extends beyond essential oil production to include applications in perfumery, pharmaceuticals, food flavoring, cosmetics, and environmental biotechnology (Chahal *et al.*, 2015). Global demand for natural essential oils has driven research into crop improvement strategies, including conventional breeding, mutation breeding, and biotechnological approaches (Smitha and Manivel, 2021; Bhardwaj *et al.*, 2020). However, the genetic improvement of these grasses faces significant challenges, including complex polyploid genomes, apomictic reproduction in some species, long breeding cycles, and limited understanding of the molecular mechanisms governing essential oil biosynthesis (Bellido *et al.*, 2024; Vimala *et al.*, 2022).

Biotechnological interventions, particularly genetic transformation and metabolic engineering, offer promising avenues for targeted improvement of these aromatic grasses (Giri and Praveena, 2015; Shabir, 2021). Recent advances in tissue culture, regeneration protocols, transcriptome sequencing, and gene discovery have laid the foundation for molecular breeding and genetic engineering approaches (Bhat *et al.*, 2023; Meena *et al.*, 2016; Devi *et al.*, 2016). This manuscript synthesizes current knowledge on genetic modification and transformation studies in *Cymbopogon* and vetiver, examining the state of the art in regeneration systems, transformation protocols, molecular characterization, and future prospects for biotechnological enhancement of these valuable aromatic grasses.

BOTANICAL AND ECONOMIC SIGNIFICANCE

Cymbopogon Species: Diversity and Applications

The genus *Cymbopogon* encompasses a wide range of aromatic grasses distributed across tropical and subtropical regions, with major centers of diversity in

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Asia and Africa (Adhikari *et al.*, 2017; Kumar *et al.*, 2009). Key commercial species include East Indian lemongrass (*C. flexuosus*), West Indian lemongrass (*C. citratus*), Java citronella (*C. winterianus*), Ceylon citronella (*C. nardus*), palmarosa (*C. martinii* var. *motia*), and gingergrass (*C. martinii* var. *sofia*) (Adeyemo and Osibote, 2023; Ng *et al.*, 2016; Padalia *et al.*, 2011). These species are distinguished by their essential oil composition, with lemongrass characterized by high citral content (70-85%), citronella by citronellal and geraniol, and palmarosa by geraniol and geranyl acetate (Gupta and Ganjewala, 2015; Kumar *et al.*, 2008; Mieso and Befa, 2020).

The essential oils of *Cymbopogon* species possess diverse biological activities, including antimicrobial, antifungal, antioxidant, insecticidal, and therapeutic properties (Ali *et al.*, 2017). Lemongrass oil, rich in citral (geranial and neral), is widely used in the fragrance industry, traditional medicine, and as a natural food preservative (Hartatie *et al.*, 2020; Bergan, 2022). Recent studies have also explored the anticancer potential of lemongrass extracts, with evidence of cytotoxic effects against colon cancer cells and enhancement of chemotherapy efficacy. The phytochemical diversity and biological activities of *Cymbopogon* essential oils have positioned these grasses as valuable crops for sustainable agriculture and natural product industries (Yadav *et al.*, 2023).

Vetiver: Multifunctional Grass for Environmental and Industrial Applications

Vetiver (*Chrysopogon zizanioides*) is a perennial grass native to India, characterized by its deep, extensive root system that can penetrate up to 3-4 meters into the soil (Nakbanpote *et al.*, 2024; Lavania and Lavania, 2009). This unique morphological feature, combined with its tolerance to extreme environmental conditions, has made vetiver a preferred species for soil and water conservation, slope stabilization, and phytoremediation of contaminated sites (Pandey and Praveen, 2020; Balangcod *et al.*, 2015). The vetiver root system produces a complex essential oil rich in sesquiterpenes, particularly vetiverol, khusimol, and α- and β-vetivone, which are highly valued in the perfume industry for their woody, earthy fragrance and fixative properties (Chahal *et al.*, 2015; Pripdeevech *et al.*, 2006).

Beyond its traditional use in perfumery, vetiver has emerged as a powerful tool for environmental biotechnology. Extensive research has demonstrated vetiver's capacity to remediate soils and waters contaminated with heavy metals (lead, cadmium, chromium, nickel, arsenic), organic pollutants (petroleum hydrocarbons, pesticides, antibiotics), and industrial effluents (Sengupta, 2014; Nakbanpote *et al.*, 2024; Nugroho *et al.*, 2021; Datta *et al.*, 2013). The grass exhibits remarkable tolerance to toxic elements through various detoxification mechanisms, including metal sequestration in roots, enzymatic degradation of organic contaminants, and symbiotic associations with pollutant-degrading microorganisms (Melato *et al.*, 2016; Das *et al.*, 2017). Recent studies have also explored vetiver's potential as a lignocellulosic feedstock for bioethanol production and biogas generation, adding value to phytoremediation applications (Sun *et al.*, 2014; Geiger *et al.*, 2019; Neve *et al.*, 2022).

In Vitro Regeneration Systems

The development of efficient in vitro regeneration systems is a prerequisite for genetic transformation and biotechnological improvement of aromatic grasses. Successful regeneration protocols have been established for multiple *Cymbopogon* species and vetiver, employing various explant types and culture conditions (Giri and Praveena, 2015; Ozias-Akins and Vasil, 1988). (George and Subramanian,1999a, 1999b, 2000)

Tissue Culture in *Cymbopogon* Species

Regeneration via Organogenesis

Organogenesis, the formation of shoots directly from explant tissues or through callus intermediates, has been successfully employed for several *Cymbopogon* species. For *C. jwarancusa*, Mehandru *et al.* developed an effective regeneration protocol using meristematic bases of spikelet explants (Mehandru *et al.*, 2014). Callogenesis was induced on Murashige and Skoog (MS) medium supplemented with 22.62 μM 2,4-dichlorophenoxyacetic acid (2,4-D), while culture multiplication was optimized with 18.1 μM 2,4-D, 4% sucrose, and 0.5% glucose. The addition of 4.65 μM kinetin enhanced regeneration, yielding approximately 40-45 plantlets per explant with 98% survival rate under greenhouse conditions (Mehandru *et al.*, 2014).

For *C. martinii* (palmarosa), Baruah and Bordoloi reported high-frequency plant regeneration through both organogenesis and embryogenesis from long-term callus cultures derived from nodal segments (Baruah and Bordoloi, 1989). Organogenesis was achieved on MS basal medium with 2 mg/L kinetin, 1 mg/L 6-benzylaminopurine (BAP), 0.2 mg/L biotin, 0.2 mg/L calcium pantothenate, and 0.1 mg/L naphthaleneacetic acid (NAA). This protocol demonstrated the feasibility of maintaining regenerative capacity in long-term callus cultures, an important consideration for genetic transformation experiments (Baruah and Bordoloi, 1989).

Somatic Embryogenesis

Somatic embryogenesis represents an alternative regeneration pathway with potential advantages for genetic transformation, including the possibility of obtaining clonal plants from single cells. For *C. citratus* (lemongrass), Quiala *et al.* developed two efficient propagation protocols: one based on organogenesis via temporary immersion systems (TIS) for shoot production, and another utilizing somatic embryogenesis (Quiala *et al.*, 2016). The somatic embryogenesis protocol involved semisolid

culture for callus formation and multiplication, followed by liquid culture in rotatory shakers and conventional bioreactors for maintaining embryogenic cultures. These methods offered fast and efficient technology for mass propagation, leveraging liquid culture and automation (Quiala *et al.*, 2016).

A rapid regeneration protocol for *C. citratus* was also reported by Lin, achieving a 90% induction rate for callus production using lamina and terminal bud explants on MS medium with 1.0-2.0 mg/L BAP and 0.2 mg/L NAA (Lin, 2008). The bud differentiation rate reached 85.6-86.7%, with a multiplication coefficient of 6. Root induction was achieved at 100% efficiency on half-strength MS medium with 0.1-0.3 mg/L NAA, and transplanted seedlings showed 100% survival after 20 days (Lin, 2008).

Regeneration Protocols for Vetiver

Callus Induction and Organogenesis

Vetiver regeneration has been achieved through multiple pathways, including organogenesis and somatic embryogenesis. Leupin *et al.* established a protocol for compact callus induction and plant regeneration from a non-flowering Java vetiver using crown and leaf slices as explants (Leupin *et al.*, 2000). Callus induction occurred on modified MS medium with 2.26 μM 2,4-D, 2.22 μM BAP, and 75 g/L sucrose. The optimized procedure regenerated plantlets on approximately 60% of crown or leaf slices, yielding up to 100 plantlets per slice (Leupin *et al.*, 2000).

Monisha *et al.* developed a high-frequency regeneration protocol for *Chrysopogon zizanioides* via both organogenesis and somatic embryogenesis (Monisha *et al.*, 2021). For organogenesis, node explants cultured on MS medium with 2.0 mg/L BAP achieved 72.2% shoot regeneration. White friable compact (WFC) callus derived from leaf or root explants on MS medium with 1.0 mg/L kinetin and 1.0 mg/L BAP showed 75.49% shoot regeneration efficiency. Somatic embryogenesis from leaf explants on MS medium with 0.5 mg/L BAP and 1.0 mg/L 2,4-D achieved 52.50% frequency, with optimal rooting at 76.97% (Monisha *et al.*, 2021).

Shoot Multiplication and Root Induction

Cedo *et al.* investigated enhanced plantlet regeneration and in vitro root production in vetiver using shoot base explants (Cedo *et al.*, 2013). Extensive shoot proliferation was maintained with 5 mg/L BAP, while root production was greatly enhanced by 0.25 mg/L NAA or 20 mg/L indole-3-butyric acid (IBA). Notably, roots induced by NAA were short and thick, whereas IBA induced long, thin, fibrous roots. Supplementation with 0.5 mg/L NAA and 0.5 mg/L kinetin significantly enhanced root proliferation (Cedo *et al.*, 2013).

Widoretno *et al.* achieved clonal propagation of vetiver through tissue culture using crown explants (Widoretno *et al.*, 2017). Shoot formation was initiated on MS medium with 2 mg/L BAP, while shoot multiplication with 3 mg/L BAP yielded an average of 126 shoots per explant. High BAP concentrations (3-5 mg/L) induced more but shorter shoots, while low BAP (1-2 mg/L) resulted in fewer but longer shoots. Plantlet regeneration involved root induction on MS medium with 1 mg/L NAA, and in vitro plants were successfully acclimatized to soil under greenhouse conditions (Widoretno *et al.*, 2017).

Somatic Embryogenesis in Vetiver

Ma *et al.* established an effective cyclic induction and regeneration system for vetiver through somatic embryogenesis (Guohua et.al. (2000), 2000). Using young leaf sheath segments and subsequently basal segments of test-tube seedlings as explants, they identified auxin as a crucial factor for inducing dedifferentiation and somatic embryo formation. Shoot formation originated from the germination of somatic embryos, induced by NAA or low concentrations of 2,4-D. This research laid a foundation for further biotechnological studies of vetiver grass (Guohua et.al. (2000), 2000).

Genetic Transformation Approaches

Despite significant progress in regeneration protocols, genetic transformation of aromatic grasses remains challenging, with limited reports of stable transgenic plants. The recalcitrance of grasses to transformation, coupled with species-specific regeneration requirements, has hindered the application of genetic engineering to *Cymbopogon* and vetiver (Giri and Praveena, 2015; Bellido *et al.*, 2024).

Agrobacterium-Mediated Transformation

Transformation of Vetiver

The most comprehensive report of genetic transformation in aromatic grasses comes from Yang *et al.*, who established an efficient Agrobacterium-mediated transformation system for vetiver (Yang *et al.*, 2008). The protocol utilized embryonic calli as target tissue, which were infected with *Agrobacterium tumefaciens* strain EHA105 harboring the plant expression vector p1301UN-otsA. The transformation procedure involved co-cultivation on callus induction medium (CIM) in the dark at 25°C for 4 days, followed by selection on shoot induction medium (SIM) with 50 mg/L hygromycin B and 500 mg/L cefotaxime (Yang *et al.*, 2008).

Key achievements of this study included an embryonic calli induction frequency of up to 96.7%, with maintained regeneration ability over two years (92.0% at 18 months, 81.6% at 24 months). The transformation efficiency, measured as the percentage of infected calli showing hygromycin B resistance, was 18%. Transient integration and expression of the transgene were confirmed by GUS (β-glucuronidase) assay. This work demonstrated the feasibility of genetic transformation in vetiver and

established a foundation for future metabolic engineering efforts (Yang *et al.*, 2008).

Transformation Attempts and Challenges

Sengupta attempted to develop hairy root culture of vetiver using *Agrobacterium rhizogenes* ATCC 15834 for enhanced phytoremediation applications (Sengupta, 2014). The protocol involved wounding and infecting the root-shoot junction of young vetiver plantlets with overnight-grown *A. rhizogenes*. However, no successful hairy root formation was observed in three independent trials, highlighting the recalcitrance of vetiver to *A. rhizogenes*-mediated transformation (Sengupta, 2014).

For *Cymbopogon* species, no published reports of successful stable genetic transformation were identified in the current literature survey. This gap represents a significant bottleneck in the biotechnological improvement of these economically important aromatic grasses. The absence of transformation protocols for *Cymbopogon* may be attributed to several factors, including the lack of optimized regeneration systems compatible with transformation procedures, genotype-specific recalcitrance, and insufficient research investment in these crops compared to major cereals (Giri and Praveena, 2015; Bellido *et al.*, 2024).

CHALLENGES AND CONSTRAINTS IN GRASS TRANSFORMATION

General Constraints in Grass Biotechnology

Genetic transformation of grasses faces multiple technical challenges that have been extensively reviewed in the context of forage and turf grasses (Liang and Wang, 2006; Wang and Ge, 2006; Giri and Praveena, 2015). Key constraints include: (1) recalcitrance to tissue culture and regeneration, with many grass species exhibiting low regeneration frequencies or loss of regenerative capacity during prolonged culture; (2) genotype dependency, where transformation efficiency varies significantly among cultivars and accessions; (3) somaclonal variation, which can lead to off-type plants with altered phenotypes; (4) low transformation efficiency, particularly for Agrobacterium-mediated methods in monocots; and (5) gene silencing, which can reduce or eliminate transgene expression in subsequent generations (Wang and Ge, 2006; Giri and Praveena, 2015).

Apomixis as a Constraint

For certain aromatic grasses, particularly some *Cymbopogon* species and related apomictic grasses, the mode of reproduction presents additional challenges for genetic improvement. Bellido *et al.* reviewed genetic transformation of apomictic grasses, highlighting both progress and constraints (Bellido *et al.*, 2024). Apomixis, the asexual reproduction through seeds, can complicate breeding efforts but also offers potential advantages for fixing hybrid vigor and maintaining desirable traits. However, the molecular complexity of apomixis and its interaction with transformation procedures remain poorly understood (Bellido *et al.*, 2024).

Recent advances in transformation of apomictic grasses have been reported for species such as *Cenchrus ciliaris* (buffelgrass), where Shashi *et al.* developed an Agrobacterium-mediated genetic engineering system (Shashi *et al.*, 2024). Such developments in related grass species provide valuable insights that may be applicable to aromatic grasses, particularly for species with apomictic reproduction (Shashi *et al.*, 2024).

Lessons from Related Grass Species

Transformation protocols developed for other grass species offer valuable templates for aromatic grass improvement. Wang and Ge reported rapid and efficient production of transgenic bermudagrass and creeping bentgrass bypassing the callus formation phase, achieving transformation through direct shoot regeneration (Wang and Ge, 2005). This approach minimized somaclonal variation and reduced the time required for transgenic plant production (Wang and Ge, 2005).

For forage grasses, Liu *et al.* demonstrated successful Agrobacterium-mediated transformation of centipedegrass (*Eremochloa ophiuroides*) (Liu *et al.*, 2012), while Faleiro *et al.* generated transgenic Napier grass (*Cenchrus purpureus*) through biolistic gene transfer using minimal expression cassettes (Faleiro *et al.*, 2021). These studies demonstrate that both Agrobacterium-mediated and biolistic transformation can be effective for grass species, depending on the specific characteristics of the target species and the optimization of culture conditions (Liu *et al.*, 2012; Faleiro *et al.*, 2021).

MOLECULAR CHARACTERIZATION AND METABOLIC ENGINEERING

Transcriptome Analysis and Gene Discovery

Transcriptome Sequencing in *Cymbopogon*

Recent advances in next-generation sequencing technologies have enabled comprehensive transcriptome analysis of aromatic grasses, providing unprecedented insights into the molecular basis of essential oil biosynthesis. Bhat *et al.* developed and analyzed de novo transcriptome assemblies of multiple genotypes of *Cymbopogon* species, revealing candidate genes involved in the biosynthesis of aromatic monoterpenes (Bhat *et al.*, 2023). This comparative transcriptomics approach identified genotype-specific expression patterns and highlighted key regulatory points in the terpenoid biosynthesis pathway (Bhat *et al.*, 2023).

Meena *et al.* performed de novo sequencing and analysis of lemongrass transcriptome, providing the first comprehensive insights into essential oil biosynthesis in aromatic grasses (Meena *et al.*, 2016). The study identified genes encoding enzymes of the methylerythritol phosphate (MEP) pathway,

mevalonate (MVA) pathway, and terpene synthases responsible for monoterpene production. This foundational work established a genomic resource for future molecular breeding and metabolic engineering efforts in lemongrass (Meena *et al.*, 2016).

For *C. winterianus* (Java citronella), Devi conducted transcriptome sequencing and characterized metabolic pathway genes (Devi, 2015). Subsequently, Devi *et al.* performed genome-wide transcriptome profiling that revealed differential gene expression in secondary metabolite pathways (Devi *et al.*, 2016). These studies identified key genes involved in citronellal and geraniol biosynthesis, including geraniol synthase, citronellol dehydrogenase, and various cytochrome P450 enzymes involved in monoterpene modification (Devi, 2015; Devi *et al.*, 2016).

Functional Characterization of Biosynthetic Genes

The functional characterization of genes involved in essential oil biosynthesis represents a critical step toward metabolic engineering of aromatic grasses. While direct genetic transformation of *Cymbopogon* species has not been reported, heterologous expression studies in model systems have validated the function of key biosynthetic enzymes. The identification of terpene synthases, prenyltransferases, and modifying enzymes from transcriptome data provides a foundation for future transgenic approaches aimed at enhancing essential oil yield or modifying oil composition (Bhat *et al.*, 2023; Meena *et al.*, 2016; Devi *et al.*, 2016).

Essential Oil Biosynthesis Pathways

Terpenoid Biosynthesis in Aromatic Grasses

Essential oils in *Cymbopogon* species are predominantly composed of monoterpenes (C10) and sesquiterpenes (C15), derived from the universal five-carbon building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In plants, these precursors are synthesized via two distinct pathways: the cytosolic mevalonate (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway (Meena *et al.*, 2016; Devi *et al.*, 2016). While the MEP pathway primarily supplies precursors for monoterpene biosynthesis in plastids, the MVA pathway provides precursors for sesquiterpene biosynthesis in the cytosol (Meena *et al.*, 2016).

The committed step in monoterpene biosynthesis is catalyzed by geranyl diphosphate synthase (GPPS), which condenses IPP and DMAPP to form geranyl diphosphate (GPP). GPP serves as the substrate for various monoterpene synthases that generate the diverse array of monoterpenes found in *Cymbopogon* essential oils. In lemongrass, citral (a mixture of geranial and neral) is the predominant component, while citronella oil is characterized by citronellal, citronellol, and geraniol (Gupta and Ganjewala, 2015; Kumar *et al.*, 2008; Padalia *et al.*, 2011).

Metabolic Engineering Strategies

Although direct metabolic engineering of aromatic grasses has not been achieved, lessons from related species provide valuable insights. Lange *et al.* demonstrated improved peppermint essential oil yield and composition through metabolic engineering, overexpressing key biosynthetic genes to enhance monoterpene production (Lange *et al.*, 2011). Similar strategies could potentially be applied to *Cymbopogon* species once efficient transformation protocols are established (Lange *et al.*, 2011).

The potential for metabolic engineering in aromatic grasses extends beyond simple overexpression of biosynthetic genes. Strategies may include: (1) upregulation of rate-limiting enzymes in the MEP or MVA pathways to increase precursor availability; (2) overexpression of specific terpene synthases to enhance production of desired monoterpenes; (3) downregulation of competing pathways to redirect carbon flux toward essential oil biosynthesis; (4) modification of regulatory genes to enhance trichome density or oil gland development; and (5) introduction of novel biosynthetic capabilities to produce non-native compounds with enhanced commercial value (Shabir, 2021; Das *et al.*, 2024; Lange *et al.*, 2011).

Conventional Genetic Improvement

While genetic transformation remains limited, conventional breeding and mutation breeding have been successfully employed for improvement of aromatic grasses.

Breeding Approaches in *Cymbopogon*

Smitha and Manivel reported genetic improvement of palmarosa (*C. martinii* var. *motia*) for herbage yield and essential oil quality through a polycross system of breeding (Smitha and Manivel, 2021). This approach exploited natural genetic variation and recombination to develop improved cultivars with enhanced agronomic performance and oil quality (Smitha and Manivel, 2021).

Phenotypic recurrent selection has been applied to citronella grass (*C. nardus*) grown in Egypt, demonstrating the effectiveness of conventional breeding for improving herb growth yield (Ibrahim and Khalid, 2013). Such approaches, while time-consuming, have proven valuable for incremental improvement of aromatic grass cultivars (Ibrahim and Khalid, 2013).

Mutation Breeding

Mutation breeding using physical and chemical mutagens has been employed to generate novel variation in aromatic grasses. Srivastava *et al.* induced mutants in the M2 generation of palmarosa and selected for enhanced essential oil yield and quality (Srivastava *et al.*, 2000). This approach successfully generated variants with improved commercial characteristics (Srivastava *et al.*, 2000).

Choudhary and Kaul reported a radiation-induced methyl-eugenol deficient mutant of *C. flexuosus*, demonstrating the potential for mutation breeding to alter essential oil composition (Choudhary and Kaul, 1979). More recently, Munda *et al.* investigated induced variations by gamma radiation and ethyl methanesulfonate (EMS) on agronomic traits, essential oil yield, and quality in Java citronella (Munda *et al.*, 2022). These studies revealed significant variation in essential oil content and composition among mutant lines, providing valuable germplasm for breeding programs (Munda *et al.*, 2022).

Ploidy Manipulation

Ploidy manipulation represents another approach for genetic improvement of aromatic grasses. Vimala *et al.* achieved lodging tolerance in *C. khasianus* through ploidy intervention, demonstrating that polyploid induction can improve agronomic characteristics (Vimala *et al.*, 2022). For vetiver, Maikami *et al.* investigated in vitro tetraploid induction to improve salt tolerance, although the relationship between ploidy level and stress tolerance requires further investigation (Maikami *et al.*, 2017).

Somaclonal Variation

Somaclonal variation, the genetic and epigenetic variation observed in plants regenerated from tissue culture, has been explored as a source of novel variation. Resmi investigated induction of somaclones in vetiver, identifying variants with altered morphological and biochemical characteristics (Resmi, 2013). While somaclonal variation can be a valuable source of genetic diversity, it also represents a challenge for maintaining genetic fidelity in micropropagation and transformation experiments (Anil *et al.*, 2018; Resmi, 2013).

Emerging Technologies and Future Directions

CRISPR/Cas9 and Genome Editing

The advent of CRISPR/Cas9 and related genome editing technologies has revolutionized plant biotechnology, offering unprecedented precision for targeted genetic modification. Recent reviews have highlighted the potential of CRISPR/Cas9-mediated genome editing in medicinal and aromatic plants (Shabir, 2021; Das *et al.*, 2024; Hefferon *et al.*, 2025). These technologies enable precise modification of endogenous genes without introducing foreign DNA, potentially addressing regulatory concerns associated with transgenic crops (Shabir, 2021).

For aromatic grasses, genome editing could be applied to: (1) knockout negative regulators of essential oil biosynthesis to enhance production; (2) modify terpene synthase genes to alter product specificity; (3) eliminate undesirable compounds from essential oil profiles; (4) enhance stress tolerance through targeted modification of stress-responsive genes; and (5) improve agronomic traits such as lodging resistance, disease resistance, and biomass yield (Shabir, 2021; Das *et al.*, 2024; Hefferon *et al.*, 2025).

However, the application of CRISPR/Cas9 to *Cymbopogon* and vetiver requires the prior establishment of efficient transformation and regeneration protocols, as well as genomic resources to identify target genes and design guide RNAs. The recent completion of the *Cymbopogon citratus* genome sequence represents an important milestone that will facilitate genome editing efforts (Chakravartty *et al.*, 2023).

Cisgenesis and Intragenesis

Cisgenesis and intragenesis represent alternative genetic engineering approaches that utilize genes from the same species or sexually compatible species, potentially offering greater public acceptance than transgenic approaches (Sarmah *et al.*, 2021). For aromatic grasses, cisgenic approaches could involve overexpression of endogenous terpene synthases or regulatory genes to enhance essential oil production without introducing foreign genetic material (Sarmah *et al.*, 2021).

Synthetic Biology and Metabolic Engineering

Advances in synthetic biology offer new possibilities for engineering complex metabolic pathways in aromatic grasses. The modular nature of terpenoid biosynthesis, with well-characterized enzymes and regulatory elements, makes these pathways amenable to synthetic biology approaches. Future efforts may involve: (1) construction of synthetic operons containing multiple biosynthetic genes under coordinated regulation; (2) introduction of novel biosynthetic modules to produce non-native terpenoids; (3) engineering of subcellular compartmentalization to optimize metabolic flux; and (4) development of inducible expression systems for controlled production of essential oils (Shabir, 2021; Das *et al.*, 2024).

Genomic Resources and Molecular Markers

The development of comprehensive genomic resources is essential for advancing molecular breeding and biotechnology in aromatic grasses. Recent studies have employed various molecular marker systems for genetic characterization and diversity analysis in *Cymbopogon* species, including RAPD, ISSR, SSR, and AFLP markers (Adhikari *et al.*, 2017; Adeyemo and Osibote, 2023; Ganjewala, 2008; Kumar *et al.*, 2009; Adhikari *et al.*, 2021). These markers facilitate germplasm characterization, cultivar identification, and marker-assisted selection in breeding programs (Adhikari *et al.*, 2017; Adeyemo and Osibote, 2023; Adhikari *et al.*, 2021).

The completion of the *Cymbopogon citratus* genome sequence by Chakravartty *et al.* represents a significant milestone, providing a reference for comparative genomics, gene discovery, and genome editing (Chakravartty *et al.*, 2023). Similar genomic resources for other *Cymbopogon* species and vetiver would greatly accelerate molecular breeding efforts (Chakravartty *et al.*, 2023).

Integration with Phytoremediation and Bioeconomy

For vetiver, future biotechnological efforts should consider the integration of essential oil production with phytoremediation and bioeconomy applications. Genetic engineering could potentially enhance both phytoremediation capacity and essential oil yield, creating dual-purpose crops that provide environmental services while generating valuable products (Nakbanpote *et al.*, 2024; Pandey and Praveen, 2020). Strategies may include: (1) overexpression of metal transporters or detoxification enzymes to enhance heavy metal accumulation; (2) introduction of genes for degradation of organic pollutants; (3) enhancement of root biomass and architecture for improved soil stabilization; and (4) optimization of essential oil biosynthesis to maintain oil quality in contaminated environments (Nakbanpote *et al.*, 2024; Pandey and Praveen, 2020).

The potential for vetiver as a lignocellulosic feedstock for biofuel production adds another dimension to its bioeconomy value (Geiger *et al.*, 2019; Neve *et al.*, 2022). Genetic modification to improve biomass yield, reduce lignin content, or enhance saccharification efficiency could increase the economic viability of vetiver-based phytoremediation systems (Geiger *et al.*, 2019; Neve *et al.*, 2022).

CONCLUSION

Genetic modification and transformation studies in aromatic grasses, particularly *Cymbopogon* and vetiver, remain at an early stage of development compared to major crop species. While efficient in vitro regeneration systems have been established for multiple species through organogenesis and somatic embryogenesis, successful stable genetic transformation has been reported only for vetiver, with an 18% transformation efficiency using Agrobacterium-mediated methods (Yang *et al.*, 2008). No published reports of stable transformation in *Cymbopogon* species were identified, representing a significant gap in the biotechnological toolkit for these economically important aromatic grasses.

Recent advances in transcriptome sequencing and molecular characterization have provided valuable insights into the genetic basis of essential oil biosynthesis, identifying candidate genes for metabolic engineering (Bhat *et al.*, 2023; Meena *et al.*, 2016; Devi, 2015; Devi *et al.*, 2016). The completion of the *Cymbopogon citratus* genome sequence marks an important milestone that will facilitate future molecular breeding and genome editing efforts (Chakravartty *et al.*, 2023). However, the translation of this molecular knowledge into practical crop improvement through genetic engineering requires the development of robust, reproducible transformation protocols for *Cymbopogon* species.

Conventional breeding and mutation breeding have demonstrated success in improving aromatic grasses, generating variants with enhanced essential oil yield, altered oil composition, and improved agronomic characteristics (Smitha and Manivel, 2021; Srivastava *et al.*, 2000; Munda *et al.*, 2022; Ibrahim and Khalid, 2013). These approaches will continue to play important roles in germplasm improvement, particularly in regions where genetically modified crops face regulatory or market acceptance challenges.

The future of aromatic grass biotechnology lies in the integration of multiple approaches: conventional breeding for near-term improvements, development of efficient transformation protocols to enable genetic engineering, application of genome editing technologies for precise modification of endogenous genes, and synthetic biology approaches for engineering complex metabolic pathways. For vetiver, the integration of essential oil production with phytoremediation and bioeconomy applications offers unique opportunities for creating multifunctional crops that provide both environmental services and valuable products (Nakbanpote *et al.*, 2024; Pandey and Praveen, 2020; Geiger *et al.*, 2019).

Key priorities for future research include: (1) development of efficient, reproducible transformation protocols for major *Cymbopogon* species; (2) functional characterization of key biosynthetic genes and regulatory elements controlling essential oil production; (3) application of genome editing technologies to modify endogenous genes without introducing foreign DNA; (4) investigation of epigenetic regulation of essential oil biosynthesis; (5) development of tissue-specific or inducible expression systems for controlled production of essential oils; and (6) integration of molecular breeding with conventional breeding to accelerate cultivar development.

The successful application of genetic modification technologies to aromatic grasses will require sustained research investment, interdisciplinary collaboration among plant biotechnologists, biochemists, breeders, and industry stakeholders, and careful attention to regulatory frameworks and public acceptance. With continued advances in genomic resources, transformation technologies, and our understanding of essential oil biosynthesis, the prospect of genetically improved aromatic grasses with enhanced productivity, quality, and environmental performance is increasingly within reach.

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REVIEW ARTICLE

MADHUCA LONGIFOLIA (MAHUA): A REVIEW OF TRADITIONAL USES, PHYTOCHEMISTRY, PHARMACOLOGICAL ACTIVITIES, TOXICOLOGY, BIOTECHNOLOGY AND THERAPEUTIC POTENTIAL

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Abstract: *Madhuca longifolia* (J.F. Gmel.) J.F. Macbr. (Sapotaceae), commonly known as Mahua or Indian Butter Tree, is a multipurpose indigenous tree widely distributed across the Indian subcontinent. It holds an important place in Ayurveda, Siddha, Unani, and tribal healthcare systems, where its flowers, leaves, bark, fruits, seeds, and seed oil have traditionally been used to treat diabetes, inflammatory disorders, respiratory and gastrointestinal ailments, skin diseases, wounds, ulcers, and rheumatism. Beyond its medicinal importance, Mahua contributes significantly to the livelihood and nutritional security of rural and tribal communities through its edible flowers, seed oil, timber, fermented products, and biodiesel potential. Growing scientific interest has highlighted *M. longifolia* as a valuable source of bioactive compounds with diverse therapeutic properties. This review summarizes together with classical Ayurvedic texts and earlier pharmacognostic studies, covering its taxonomy, botanical characteristics, distribution, ethnomedicinal importance, nutritional composition, phytochemistry, pharmacognosy, pharmacological activities, toxicology, biotechnology, and pharmaceutical applications. Phytochemical studies have identified numerous bioactive constituents, including triterpenoids, saponins, flavonoids, phenolic compounds, phytosterols, fatty acids, tannins, glycosides, and vitamins, which are responsible for its wide range of biological activities. Experimental studies have demonstrated significant antioxidant, anti-inflammatory, antimicrobial, antidiabetic, hepatoprotective, gastroprotective, wound-healing, analgesic, anticancer, and immunomodulatory properties. Recent advances in metabolomics, chromatographic fingerprinting, tissue culture, nanotechnology, and phytopharmaceutical research have further strengthened its medicinal and industrial potential. However, despite encouraging preclinical findings, standardized extracts, pharmacokinetic investigations, long-term safety assessments, and well-designed clinical trials remain limited. Future multidisciplinary research integrating phytochemistry, pharmacology, biotechnology, and clinical sciences is essential to develop safe, standardized, and evidence-based Mahua-derived phytopharmaceuticals for the management of chronic diseases.

Keywords: *Madhuca longifolia*, Mahua, Sapotaceae, Ethnomedicine, Phytochemistry, Medicinal plant

INTRODUCTION

Medicinal plants have served as the cornerstone of traditional healthcare systems for thousands of years and continue to provide valuable therapeutic agents for the prevention and treatment of numerous human diseases (Tomar, 2007; Tomar, 2009; Tomar, 2019; Tomar, 2020; Tomar, 2022, Tomar, 2023 and Sharma *et al.*, 2025). According to the World Health Organization (WHO), nearly 80% of the global population relies, either wholly or partially, on traditional herbal medicines for primary healthcare, particularly in developing countries where medicinal plants remain accessible, affordable, and culturally acceptable (World Health Organization,2013). Increasing concerns regarding antimicrobial resistance, chronic non-communicable diseases, adverse effects of synthetic drugs, and the growing demand for natural therapeutics have renewed worldwide interest in medicinal plants as promising sources of novel bioactive compounds and phytopharmaceuticals.

The family Sapotaceae comprises approximately 65 genera and over 1,250 species distributed predominantly throughout tropical and subtropical regions of Asia, Africa, Australia, and the Americas (Pennington, 1991). Members of this family are well known for their latex-bearing tissues, edible fruits, nutrient-rich seeds, and abundance of biologically active secondary metabolites such as triterpenoids, flavonoids, saponins, phenolic compounds, sterols, and fatty acids. Among them, *Madhuca longifolia* (J.F. Gmel.) J.F. Macbr. is one of the most valuable indigenous tree species of the Indian subcontinent because of its exceptional medicinal, nutritional, ecological, cultural, and economic importance (Khare, 2007).

Commonly known as Mahua, Mowra, Illuppai, or Indian Butter Tree, *M. longifolia* is a medium- to large-sized deciduous tree widely distributed throughout central, western, eastern, and peninsular

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India, extending into Nepal, Sri Lanka, Myanmar, and parts of Southeast Asia. The species thrives in tropical dry deciduous forests and semi-arid ecosystems up to an altitude of approximately 1,200 m above sea level. Owing to its remarkable adaptability to harsh environmental conditions, drought tolerance, and multipurpose utility, Mahua has been regarded as the "Tree of Life" among many indigenous and tribal communities of India. Nearly every component of the tree—including flowers, fruits, seeds, bark, leaves, latex, and wood—is utilized for food, traditional medicine, livestock feed, fuel, timber, biodiesel production, cosmetics, soap manufacturing, and numerous household applications (Orwa *et al.*, 2009).

In the Ayurvedic system of medicine, Mahua is known as Madhuka and is described as possessing Madhura (sweet) and Kashaya (astringent) rasa, Guru (heavy) and Snigdha (unctuous) guna, Sheeta (cooling) virya, and Madhura vipaka. It is traditionally prescribed as a Rasayana, Balya, Vrishya, Brimhana, Raktapittahara, and Vranahara drug for promoting vitality, improving reproductive health, relieving thirst and burning sensations, healing wounds, and managing respiratory and gastrointestinal disorders. Flowers are used as nutritive tonics and expectorants; bark is valued for its astringent and anti-inflammatory properties; leaves are applied to skin diseases and wounds; while seed oil is widely employed for rheumatism, eczema, chronic ulcers, and other dermatological conditions (Nadkarni, 1954; Khare, 2007).

Beyond its medicinal significance, Mahua has substantial nutritional and industrial importance. The carbohydrate-rich flowers are consumed fresh or dried and are widely utilized in the preparation of traditional fermented beverages, syrups, confectionery products, vinegar, and livestock feed. Seeds contain 35–50% edible oil rich in oleic, stearic, palmitic, and linoleic acids, which is extensively used in food processing, biodiesel production, cosmetics, soap manufacture, lubricants, and pharmaceutical formulations. The residual seed cake serves as an organic manure, biofertilizer, and botanical pesticide, thereby contributing to sustainable agriculture and circular bioeconomy.

Phytochemical investigations carried out during the past several decades have revealed an extraordinary diversity of secondary metabolites, including triterpenoids (α-amyrin, β-amyrin, lupeol, betulinic acid, oleanolic acid), triterpenoid saponins, flavonoids (quercetin, myricetin, dihydroquercetin), phenolic acids, phytosterols, glycosides, tannins, carbohydrates, amino acids, vitamins, carotenoids, and fatty acids. These compounds have been associated with a broad range of pharmacological activities including antioxidant, anti-inflammatory, antimicrobial, antidiabetic, hepatoprotective, nephroprotective, gastroprotective, analgesic, wound-healing, immunomodulatory, cardioprotective, neuroprotective, anticancer, antiulcer, spermicidal, insecticidal, and antiviral properties (Patel & Naik, 2010; Grover *et al.*, 2022).

Recent advances in analytical chemistry, metabolomics, high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC–MS/MS), nuclear magnetic resonance (NMR), molecular docking, DNA barcoding, tissue culture, and nanotechnology have considerably expanded scientific understanding of *M. longifolia*. These modern approaches have facilitated the identification of novel bioactive molecules, quality-control markers, and potential therapeutic targets while supporting the development of standardized herbal formulations and phytopharmaceutical products.

Despite its extensive traditional use and increasing scientific recognition, information concerning the taxonomy, ethnomedicine, phytochemistry, pharmacognosy, pharmacological properties, toxicology, biotechnology, and pharmaceutical applications of *M. longifolia* remains fragmented across ethnobotanical reports, pharmacological investigations, phytochemical studies, and review articles. Furthermore, substantial research published during the last decade has not been comprehensively integrated with classical knowledge.

Therefore, the present review critically compiles and evaluates the available literature published between 2000 and 2025 together with historical reports to provide an updated overview of the botanical characteristics, geographical distribution, traditional uses, nutritional composition, phytochemical profile, pharmacognostic standards, pharmacological activities, toxicological aspects, biotechnology, and therapeutic potential of *Madhuca longifolia*. The review also highlights current research gaps, future perspectives, and opportunities for developing standardized, safe, and evidence-based phytopharmaceuticals from this culturally and economically important medicinal tree.

Botanical Description and Taxonomy

Madhuca longifolia (J.F. Gmel.) J.F. Macbr. is a large multipurpose deciduous tree belonging to the family Sapotaceae, valued for its nutritional, medicinal, ecological, and socioeconomic importance (Tomar, 2024). The species has been recognized in Ayurvedic literature since ancient times under the Sanskrit name Madhuka, where it is described as a rejuvenating (Rasayana) plant possessing cooling, nutritive, tonic, and wound-healing properties (Nadkarni, 1954). Historically, the species has also been reported under the synonyms *Bassia longifolia* Koenig ex L., *Bassia latifolia* Roxb., *Madhuca indica* J.F. Gmel., and *Madhuca latifolia* (Roxb.) A. Chev., reflecting changes in botanical nomenclature over time (Pennington, 1991; Plants of the World Online [POWO], 2025).

Modern taxonomic treatments recognize two botanical varieties, namely *Madhuca longifolia* var. *longifolia*, predominantly distributed in southern India, and *Madhuca longifolia* var. *latifolia* (Roxb.) A. Chev., which occurs mainly in central and northern India (POWO, 2025).

Taxonomic Classification (According to the Linnaean System)

Taxonomic Rank	Classification
Kingdom	Plantae
Division	Magnoliophyta (Angiosperms)
Class	Magnoliopsida (Dicotyledons)
Subclass	Dilleniidae
Order	Ebenales *(currently placed in Ericales under APG IV)*
Family	Sapotaceae
Genus	*Madhuca* J.F. Gmel.
Species	*Madhuca longifolia* (J.F. Gmel.) J.F. Macbr.

Accepted scientific name: *Madhuca longifolia* (J.F. Gmel.) J.F. Macbr.

Important synonyms: *Bassia longifolia* Koenig ex L.; *Bassia latifolia* Roxb.; *Madhuca indica* J.F. Gmel.; *Madhuca latifolia* (Roxb.) A. Chev (Pennington, 1991; Khare, 2007; POWO, 2025).

Vernacular Names

Madhuca longifolia (J.F. Gmel.) J.F. Macbr. is widely recognized throughout the Indian subcontinent under numerous vernacular names, reflecting its extensive geographical distribution and profound cultural, medicinal, and socioeconomic importance. In the Ayurvedic system of medicine, the species is known as Madhuka, with several Sanskrit synonyms including Gudapushpa, Madhudruma, Madhushrava, and Deerghapatra, each describing its distinctive botanical characteristics or therapeutic properties (Nadkarni, 1954; Khare, 2007). In English, it is commonly referred to as Mahua, Indian Butter Tree, or Butter Tree, owing to the edible fat extracted from its seeds. Across different regions of India, the tree is known by various local names, including Mahua or Mohwa in Hindi, Moha or Mohwa in Marathi, Mahudo in Gujarati, Mahua in Bengali, Illuppai in Tamil, Ippa or Ippachettu in Telugu, Hippe in Kannada, and Irippa in Malayalam. The diversity of vernacular names highlights the widespread utilization of *M. longifolia* in traditional medicine, food, religious practices, and rural livelihoods throughout India, emphasizing its significance as one of the country's most valuable indigenous multipurpose tree species (Orwa *et al.*, 2009; Patel & Naik, 2010).

Botanical Description

Madhuca longifolia is a medium- to large-sized deciduous tree attaining a height of 15–20 m, occasionally reaching 25 m under favourable environmental conditions (Tomar,2024). Historical botanical descriptions characterize the tree as possessing a short stout bole with a broad, dense, rounded crown and thick spreading branches (Nadkarni, 1954). The bark is dark grey to brown, rough, longitudinally fissured, and exudes milky latex upon injury. The inner bark is reddish-brown and rich in tannins, explaining its traditional use as an astringent in Ayurveda (Raj & Patel, 1978).

Leaves are simple, alternate, coriaceous, elliptic-oblong to obovate, measuring approximately 10–25 cm long and 5–12 cm wide. Young foliage is reddish-copper in colour before gradually becoming glossy dark green at maturity. Leaves are clustered near the terminal portions of branches and remain persistent for most of the year (Orwa *et al.*, 2009).

Flowering generally occurs between February and April, coinciding with seasonal leaf fall. Flowers are fleshy, cream to pale yellow, fragrant, bisexual, and borne in dense fascicles on leafless branches. They possess a persistent calyx and a tubular corolla with numerous stamens. Flowers are exceptionally rich in fermentable sugars, carbohydrates, vitamins, amino acids, and minerals, making them valuable both nutritionally and medicinally (Patel & Naik, 2010). Traditional literature describes the flowers as cooling, demulcent, tonic, expectorant, nutritive, stimulant, and useful in piles and respiratory ailments (Nadkarni, 1954; Ahluwalia, 1968).

The fruit is an ovoid to ellipsoid fleshy berry measuring 2–6 cm in length, containing one to four smooth, shiny brown seeds embedded in sweet pulp. Seeds contain approximately 35–50% fixed oil, rich in oleic, stearic, palmitic, and linoleic acids, widely utilized in food industries, cosmetics, soap manufacture, pharmaceuticals, and biodiesel production (Ramadan *et al.*, 2006).

The tree develops a deep taproot system with extensive lateral roots that confer remarkable drought tolerance and adaptability to marginal soils, making it highly suitable for agroforestry and ecological restoration programmes (Orwa *et al.*, 2009).

DISTRIBUTION AND HABITAT

Madhuca longifolia is indigenous to the Indian subcontinent and is naturally distributed throughout India, Nepal, Bangladesh, Sri Lanka, and Myanmar, with limited cultivation in several tropical countries because of its nutritional and economic value (Orwa *et al.*, 2009; POWO, 2025).

Historical botanical literature describes Mahua as a medium- to large-sized deciduous tree occurring throughout the greater parts of India up to an altitude of about 1,200 m, especially in dry deciduous forests (Nadkarni, 1954). The species is particularly abundant in Madhya Pradesh, Maharashtra, Chhattisgarh, Jharkhand, Odisha, Gujarat, Rajasthan, Uttar Pradesh, Bihar, Telangana, Andhra Pradesh, Karnataka, Tamil Nadu, and West Bengal, where it constitutes an important component of tribal ecosystems and rural livelihoods (Patel & Naik, 2010).

The two botanical varieties exhibit distinct geographical distributions. *Madhuca longifolia* var. *latifolia* predominates in central and northern India, whereas *M. longifolia* var. *longifolia* is mainly confined to peninsular India, particularly Karnataka, Tamil Nadu, Kerala, Telangana, and Andhra Pradesh (POWO, 2025).

The species grows naturally from sea level to approximately 1,200 m elevation under tropical and subtropical climates with annual rainfall ranging between 500 and 1,500 mm and temperatures from 5°C to 46°C. Mature trees exhibit exceptional drought tolerance because of their extensive root system and ability to withstand prolonged moisture stress (Orwa *et al.*, 2009).

Madhuca longifolia thrives on deep, well-drained loamy, sandy-loam, lateritic, and black cotton soils, although it can also establish successfully on degraded lands and gravelly substrates. The species tolerates soil pH ranging from 5.5 to 8.5 and is frequently encountered in dry deciduous forests, village commons, sacred groves, agricultural field boundaries, and agroforestry systems.

Ecologically, Mahua functions as a keystone species supporting pollinating insects, honeybees, birds, bats, and mammals through its nectar-rich flowers and nutritious fruits. The annual leaf litter contributes significantly to nutrient cycling and soil fertility, while its extensive canopy enhances carbon sequestration and biodiversity conservation. Because of its outstanding ecological adaptability and socioeconomic importance, Mahua is often referred to as the "Tree of Life" or "Kalpavriksha of Tribal India", providing food, medicine, fodder, fuel, timber, and sustainable income to millions of forest-dependent communities (Patel & Naik, 2010).

TRADITIONAL AND ETHNOMEDICINAL USES

Madhuca longifolia (J.F. Gmel.) J.F. Macbr. has occupied a prominent position in the traditional healthcare systems of India for centuries and is extensively documented in Ayurveda, Siddha, Unani, and various indigenous tribal medicinal practices. In Ayurveda, the plant is known as Madhuka and is described as possessing Madhura (sweet) and Kashaya (astringent) rasa, Guru (heavy) and Snigdha (unctuous) guna, Sheeta (cooling) virya, and Madhura vipaka. It is traditionally classified as Balya (strength promoting), Brimhana (body nourishing), Rasayana (rejuvenative), Vrishya (aphrodisiac), Shukrakara, Raktapittahara, Trishnashamana, Vranahara, and Kshatakshayahara, indicating its usefulness in improving vitality, wound healing, reproductive health, and chronic debilitating disorders (Nadkarni, 1954; Khare, 2007).

Almost every part of the plant, including flowers, bark, leaves, fruits, seeds, seed oil, and latex, is utilized therapeutically. The flowers are considered cooling, demulcent, nutritive, tonic, expectorant, stimulant, and appetizer. They are traditionally prescribed for cough, bronchitis, asthma, thirst, burning sensation, emaciation, general debility, bleeding disorders, piles, and chronic constipation. Dried flowers are widely consumed as a restorative food and are fermented to prepare traditional beverages among tribal communities of central and southern India (Nadkarni, 1954; Patel & Naik, 2010; Dhale & Patil, 2024).

The bark possesses pronounced astringent, anti-inflammatory, and antimicrobial properties. Bark decoctions are traditionally administered for diabetes mellitus, diarrhoea, dysentery, bleeding gums, rheumatism, itching, skin diseases, ulcers, and chronic wounds. Tribal healers also prescribe bark preparations for fever and inflammatory conditions (Raj & Patel, 1978; Khare, 2007).

Leaves are extensively employed as topical remedies. Fresh leaves are applied over wounds, burns, eczema, boils, fractures, sprains, and inflammatory swellings. Warm leaf poultices are used to relieve joint pain, rheumatism, and muscular inflammation, whereas leaf decoctions are administered internally for respiratory ailments and skin disorders. The latex is occasionally applied to cracked skin and chronic ulcers because of its wound-healing properties (Nadkarni, 1954).

The seeds yield Mahua butter, a fixed oil that has been used traditionally as a laxative, emollient, analgesic, and anti-inflammatory agent. Seed oil is externally applied for eczema, psoriasis, scabies, rheumatism, arthritis, chronic ulcers, cracked heels, and various dermatological disorders. Internally, it has been used in small quantities for constipation and as a nutritive fat in rural communities (Raj & Patel, 1978; Khare, 2007).

Ethnobotanical investigations conducted across Maharashtra, Madhya Pradesh, Chhattisgarh, Jharkhand, Odisha, Gujarat, Rajasthan, and Telangana have documented the continued dependence of tribal communities such as the Gond, Baiga, Bhil, Kol, Santhal, Oraon, and Koya tribes on *M. longifolia* for primary healthcare, nutrition, and livelihood. Flowers are consumed fresh or dried as food, seeds are processed for edible and medicinal oil, while bark and leaves remain important components of traditional herbal formulations for gastrointestinal disorders, diabetes, respiratory diseases, reproductive ailments, and skin infections (Jain, 1991; Orwa *et al.*, 2009; Singh *et al.*, 2021).

Recent pharmacological investigations strongly support many of these traditional claims by demonstrating antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, antidiabetic, analgesic, wound-healing, gastroprotective, and immunomodulatory activities, thereby validating the ethnomedicinal importance of *Madhuca longifolia* (Grover *et al.*, 2022; Singh *et al.*, 2023).

NUTRITIONAL COMPOSITION

Madhuca longifolia is regarded as one of the most nutritionally valuable forest tree species of India. Its flowers, fruits, and seeds constitute important dietary resources for rural and tribal populations, particularly during periods of food scarcity. The flowers are highly nutritious because of their high carbohydrate content, making them an excellent source of dietary energy. Fresh flowers contain approximately 60–70% fermentable sugars, mainly glucose, fructose, and sucrose, together with proteins, amino acids, vitamins, minerals, dietary fibre, and natural antioxidants (Gopalan *et al.*, 2019; Patel & Naik, 2010).

The flowers are rich in essential minerals such as calcium, phosphorus, potassium, magnesium, iron, zinc, and copper. Earlier nutritional analyses reported approximately 45 mg calcium, 22 mg phosphorus, 40 mg iron, 1.1 mg vitamin C, and 307 µg carotene per 100 g of edible flowers (Gopalan *et al.*, 1984). In addition, they contain B-complex vitamins, amino acids, and phenolic compounds that contribute to their nutritional and antioxidant value.

The seeds contain 35–50% fixed oil, commonly referred to as Mahua butter. The oil is composed predominantly of oleic acid (40–50%), stearic acid (20–25%), palmitic acid (20–25%), linoleic acid (8–14%), and smaller quantities of arachidic, behenic, and myristic acids. This fatty acid profile imparts excellent nutritional as well as industrial value to the seed oil, making it suitable for edible applications after refining, pharmaceutical preparations, cosmetics, soap manufacture, and biodiesel production (Ramadan *et al.*, 2006; Patel & Naik, 2010).

The flowers also contain appreciable quantities of reducing sugars, proteins, dietary fibre, organic acids, flavonoids, and phenolic compounds, which collectively contribute to their antioxidant capacity and health-promoting properties. Recent metabolomic studies have further identified several bioactive phytochemicals that enhance the nutraceutical potential of Mahua flowers, supporting their use in functional foods, fermented beverages, syrups, confectionery products, bakery formulations, and herbal health supplements (Grover *et al.*, 2022).

Mahua seed cake, obtained after oil extraction, is rich in proteins, carbohydrates, and minerals. Although the presence of saponins limits its direct human consumption, detoxified seed cake is increasingly being explored as a protein-rich livestock feed, organic fertilizer, biofertilizer, and botanical pesticide. The flowers are also processed into value-added products including jaggery, vinegar, wine, ethanol, nectar, squash, candies, and fermented beverages, significantly contributing to the rural bioeconomy and food security (Patel & Naik, 2010; Singh *et al.*, 2023).

The remarkable nutritional composition of *Madhuca longifolia*, combined with its abundance of phytochemicals and traditional medicinal value, has attracted increasing attention toward its development as a functional food, nutraceutical ingredient, and phytopharmaceutical resource for improving human health and nutrition.

PHYTOCHEMISTRY

Madhuca longifolia (J.F. Gmel.) J.F. Macbr. is a rich source of structurally diverse phytochemicals distributed throughout its flowers, leaves, bark, fruits, seeds, and seed oil. Since the early phytochemical investigations of the 1950s and 1960s, numerous studies have demonstrated that the medicinal properties of the plant are attributed to the presence of triterpenoids, triterpenoid saponins, flavonoids, phenolic compounds, phytosterols, glycosides, fatty acids, tannins, carbohydrates, amino acids, vitamins, and volatile constituents (Mitra & Awasthi, 1962; Awasthi & Mitra, 1967, 1968). Recent investigations employing HPLC, GC–MS, LC–MS/MS, NMR spectroscopy, and metabolomics have further expanded the phytochemical profile of the species and identified several pharmacologically active metabolites (Grover *et al.*, 2022; Singh *et al.*, 2023).

The flowers are rich in reducing sugars (glucose, fructose, and sucrose), proteins, amino acids, vitamins, carotenoids, minerals, flavonoids, and phenolic acids, making them nutritionally valuable and responsible for their antioxidant activity (Patel & Naik, 2010). Phytochemical screening has revealed the presence of quercetin derivatives, myricetin, gallic acid, chlorogenic acid, caffeic acid, catechin, and rutin, which contribute to the antioxidant, anti-inflammatory, and hepatoprotective activities of the flowers (Grover *et al.*, 2022).

Leaves contain abundant flavonoids, including myricetin, quercetin, myricitrin, quercitrin, myricetin-3-*O*-L-rhamnoside, myricetin-3-arabinoside, and quercetin-3-galactoside, together with tannins, saponins, phenolic compounds, and xanthophyll pigments (Subramanian & Nair, 1972, 1973). Triterpenoids such as oleanolic acid palmitate, 3-β-caproxy-olea-12-en-28-ol, and β-sitosterol glucoside have also been isolated from leaf extracts (Bhatnagar *et al.*, 1972).

The bark is particularly rich in triterpenoids and phytosterols. Phytochemical investigations have identified lupeol acetate, β-amyrin acetate, α-spinasterol, erythrodiol monocaprylate, betulinic acid, oleanolic acid caprylate, and β-sitosterol glucoside. The aqueous fraction additionally contains monosaccharides such as D-glucose, D-galactose, D-xylose, and L-rhamnose, which may contribute to its biological activities (Awasthi & Mitra, 1968). These constituents are largely responsible for the anti-

inflammatory, antimicrobial, hepatoprotective, and wound-healing properties of the bark.

Seeds are chemically characterized by a high content of triterpenoid saponins, fixed oil, phytosterols, and glycosides. Two major saponins, designated Saponin A and Saponin B, were isolated and structurally characterized from defatted seeds. These compounds possess bassic acid as the principal sapogenin and have demonstrated spermicidal and antimicrobial activities (Hariharan *et al.*, 1972; Banerji *et al.*, 1979). The seed oil contains approximately 35–50% lipids, consisting mainly of oleic acid, stearic acid, palmitic acid, linoleic acid, and smaller amounts of arachidic and behenic acids, making it suitable for pharmaceutical, cosmetic, and nutraceutical applications (Ramadan *et al.*, 2006).

The fruits contain sugars, phenolic compounds, flavonoids, vitamins, and carotenoids, whereas latex contains proteins, terpenoids, and other bioactive metabolites involved in wound healing and plant defense.

Recent metabolomic analyses have identified numerous volatile compounds, sterols, tocopherols, and polyphenolic constituents that further strengthen the antioxidant, anti-inflammatory, antidiabetic, antimicrobial, and anticancer potential of *Madhuca longifolia*. Advances in chromatographic fingerprinting and metabolomic profiling have also facilitated the establishment of quality-control parameters for herbal formulations prepared from Mahua (Grover *et al.*, 2022; Singh *et al.*, 2023).

Overall, the remarkable diversity of phytochemicals present in *M. longifolia* provides the scientific basis for its broad spectrum of traditional medicinal uses and experimentally validated pharmacological activities.

PHARMACOGNOSY

Pharmacognostic evaluation plays a crucial role in the authentication, quality control, and standardization of medicinal plants. *Madhuca longifolia* has been extensively investigated for its macroscopic, microscopic, physicochemical, and phytochemical characteristics, which serve as reliable diagnostic parameters for identification of crude drugs and herbal formulations (Anonymous, 2001; Khare, 2007).

Macroscopically, *M. longifolia* is a medium- to large-sized deciduous tree with a short, stout trunk and a broad, spreading crown. The bark is dark grey to brown, rough, longitudinally fissured, and exudes milky latex upon injury. Leaves are simple, alternate, coriaceous, elliptic-oblong to obovate, measuring approximately 10–25 cm in length, with entire margins and acute to obtuse apices. Flowers are fleshy, cream to pale yellow, fragrant, bisexual, and borne in dense fascicles on leafless branches. Fruits are fleshy berries containing one to four smooth brown seeds rich in fixed oil (Nadkarni, 1954; Orwa *et al.*, 2009).

Microscopic examination of the bark reveals a multilayered cork composed of rectangular suberized cells followed by a broad cortex containing parenchymatous tissues with abundant tannin cells, stone cells, and laticiferous canals. Calcium oxalate crystals, starch grains, and sclerenchymatous fibres are frequently observed. Secondary phloem is well developed with medullary rays and numerous secretory cells containing phenolic substances (Anonymous, 2001).

Leaf microscopy shows a dorsiventral lamina with a single-layered epidermis covered by a thick cuticle. The mesophyll is differentiated into one to two layers of palisade cells and several layers of spongy parenchyma. Numerous collateral vascular bundles, laticifers, calcium oxalate crystals, and unicellular covering trichomes are present. Stomata are predominantly paracytic and occur mainly on the lower epidermis, indicating a hypostomatic leaf (Khare, 2007).

Powder microscopy of bark and leaves exhibits characteristic fragments of cork cells, lignified fibres, sclereids, spiral and pitted vessels, starch grains, tannin-containing cells, oil globules, calcium oxalate crystals, and pollen grains, which provide important diagnostic markers for authentication and detection of adulteration.

Physicochemical evaluation has reported acceptable ranges for total ash, acid-insoluble ash, water-soluble ash, moisture content, alcohol-soluble extractive, and water-soluble extractive values according to pharmacopoeial standards. Fluorescence analysis under visible and ultraviolet light following treatment with different chemical reagents has also been employed for quality assessment (Anonymous, 2001).

Preliminary phytochemical screening of different plant parts consistently demonstrates the presence of alkaloids (trace), flavonoids, triterpenoids, saponins, tannins, glycosides, steroids, carbohydrates, proteins, amino acids, fixed oils, and phenolic compounds, supporting the therapeutic potential of the species (Khare, 2007; Grover *et al.*, 2022).

Modern pharmacognostic approaches involving High-Performance Thin-Layer Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), Gas Chromatography–Mass Spectrometry (GC–MS), Liquid Chromatography–Mass Spectrometry (LC–MS/MS), Fourier Transform Infrared Spectroscopy (FTIR), DNA barcoding, and metabolomic fingerprinting have significantly improved authentication and standardization of *Madhuca longifolia*. These analytical tools facilitate identification of marker compounds such as β-sitosterol, lupeol, betulinic acid, oleanolic acid, quercetin, myricetin, and triterpenoid saponins, ensuring the quality, efficacy, and safety of herbal preparations intended for

pharmaceutical applications (Grover *et al.*, 2022; Singh *et al.*, 2023).

PHARMACOLOGICAL ACTIVITIES

The diverse ethnomedicinal applications of *Madhuca longifolia* (J.F. Gmel.) J.F. Macbr. have stimulated extensive pharmacological investigations over the past several decades. Experimental studies using crude extracts, solvent fractions, purified phytochemicals, and isolated compounds have demonstrated a broad spectrum of biological activities that largely validate its traditional medicinal uses. These pharmacological effects are primarily attributed to the synergistic action of triterpenoids, triterpenoid saponins, flavonoids, phenolic compounds, phytosterols, fatty acids, glycosides, and tannins present in different parts of the plant (Khare, 2007; Grover *et al.*, 2022).

Antioxidant Activity

Several in vitro and in vivo studies have demonstrated that flower, leaf, bark, and seed extracts possess potent antioxidant activity by scavenging reactive oxygen species (ROS), inhibiting lipid peroxidation, and enhancing endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase. The antioxidant potential is mainly attributed to flavonoids, phenolic acids, quercetin, myricetin, and tannins (Patel & Naik, 2010; Grover *et al.*, 2022).

Anti-inflammatory and Analgesic Activity

Traditional use of Mahua in rheumatism and inflammatory disorders has been supported by experimental studies demonstrating significant inhibition of carrageenan-induced paw edema, cotton pellet granuloma, and inflammatory mediators including cyclooxygenase (COX) and nitric oxide. Triterpenoids such as lupeol, β-amyrin, oleanolic acid, and betulinic acid contribute significantly to these effects. Extracts also exhibit peripheral analgesic activity in animal models, supporting their traditional use in pain management (Khare, 2007; Singh *et al.*, 2023).

Antidiabetic Activity

Methanolic and aqueous extracts of bark, leaves, flowers, and seeds have demonstrated significant hypoglycemic effects in streptozotocin- and alloxan-induced diabetic experimental models. Treatment resulted in reduced blood glucose, improved insulin sensitivity, restoration of pancreatic β-cell function, and decreased oxidative stress. These effects have been attributed to flavonoids, phenolic compounds, triterpenoids, and saponins (Raj & Patel, 1978; Grover *et al.*, 2022).

Antimicrobial Activity

Extracts prepared from flowers, leaves, stem bark, and roots exhibit broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria including *Bacillus subtilis*, *Bacillus anthracis*, *Staphylococcus aureus*, *Salmonella paratyphi*, *Vibrio cholerae*, and *Xanthomonas campestris*. Antifungal activity against several pathogenic fungi has also been reported. Earlier investigations demonstrated antibacterial activity of methanolic extracts against medically important microorganisms (Trivedi *et al.*, 1980), while recent studies have confirmed these findings using modern microbiological techniques (Grover *et al.*, 2022).

Hepatoprotective Activity

Experimental studies have demonstrated that flower and bark extracts protect hepatic tissues against carbon tetrachloride-, paracetamol-, and alcohol-induced liver damage by reducing lipid peroxidation, restoring hepatic antioxidant enzymes, and improving serum biochemical markers including AST, ALT, and ALP. Hepatoprotective activity is mainly associated with flavonoids, phenolic compounds, and triterpenoids (Singh *et al.*, 2023).

Gastroprotective and Antiulcer Activity

Traditional use of Mahua flowers for gastrointestinal disorders has been supported by experimental evidence demonstrating significant reduction in gastric ulcer index, gastric acidity, and mucosal damage in ethanol- and indomethacin-induced ulcer models. The mucosal protective effects are attributed to flavonoids, mucilage, tannins, and antioxidant constituents that enhance gastric mucus secretion and reduce oxidative injury (Khare, 2007; Grover *et al.*, 2022).

Wound-Healing Activity

Leaf, bark, and seed oil preparations have been traditionally applied to wounds, burns, ulcers, and skin infections. Scientific investigations have shown accelerated wound contraction, increased collagen synthesis, enhanced epithelialization, and improved tensile strength in excision and incision wound models. These effects are associated with antimicrobial, antioxidant, and anti-inflammatory phytochemicals (Nadkarni, 1954; Singh *et al.*, 2023).

Anticancer Activity

Recent in vitro investigations indicate that extracts of *M. longifolia* possess cytotoxic activity against selected human cancer cell lines by inducing apoptosis, inhibiting cell proliferation, and modulating oxidative stress pathways. Betulinic acid, oleanolic acid, lupeol, and phenolic compounds are considered the principal anticancer constituents. Nevertheless, further mechanistic studies and clinical investigations are required before therapeutic application (Grover *et al.*, 2022).

Spermicidal and Contraceptive Activity

One of the most extensively investigated pharmacological properties of *M. longifolia* is its spermicidal activity. Triterpenoid saponins isolated from seeds demonstrated remarkable spermicidal activity against human semen at low concentrations. Saponin A and Saponin B, possessing bassic acid as the principal sapogenin, exhibited significant contraceptive potential without marked

cardiovascular effects (Setty *et al.*, 1976; Banerji *et al.*, 1979, 1981).

Other Pharmacological Activities

Various investigations have also reported hypotensive, antiviral, immunomodulatory, cardioprotective, nephroprotective, neuroprotective, antihelmintic, insecticidal, and nematicidal activities. Earlier pharmacological screening demonstrated hypotensive effects of stem bark extracts in cats and dogs (Bhakuni *et al.*, 1969), antiviral activity against Potato virus X (Singh & Singh, 1972), and insecticidal properties against houseflies (Atal *et al.*, 1978). Furthermore, Mahua seed cake exhibits nematicidal activity against important agricultural nematodes, suggesting potential applications in sustainable agriculture (Alam *et al.*, 1982).

Overall, accumulating pharmacological evidence strongly supports the traditional therapeutic claims associated with *M. longifolia*. However, most investigations remain preclinical, emphasizing the need for standardized formulations, pharmacokinetic studies, and well-designed clinical trials to establish therapeutic efficacy and safety in humans.

TOXICOLOGY AND SAFETY

Although *Madhuca longifolia* has been consumed safely for centuries as food and traditional medicine, toxicological evaluation remains essential for ensuring its safe therapeutic application. Available evidence indicates that edible flowers, properly processed seed oil, and traditionally prescribed doses of herbal preparations are generally considered safe. Nevertheless, certain plant constituents, particularly triterpenoid saponins present in seeds and seed cake, exhibit dose-dependent toxicity and therefore require appropriate processing before medicinal or dietary use (Mulky & Gandhi, 1977; Banerji *et al.*, 1981).

Acute toxicity studies on 50% alcoholic stem bark extract demonstrated an intraperitoneal LD_{50} of approximately 1000 mg/kg in albino mice, indicating relatively low acute toxicity under experimental conditions (Bhakuni *et al.*, 1969). Similarly, ethanolic extracts of aerial parts exhibited LD_{50} values greater than 1000 mg/kg, suggesting a wide margin of safety for crude extracts when administered in recommended doses (Dhar *et al.*, 1974).

Triterpenoid saponins isolated from Mahua seeds have received considerable toxicological attention because of their spermicidal activity. Experimental investigations revealed that these saponins possess minimal cardiovascular and haemolytic activity at pharmacologically effective concentrations but become highly toxic when administered parenterally at high doses. Mulky and Gandhi (1977) reported that the acute toxicity of seed saponins following intravenous or intraperitoneal administration was substantially greater than after oral administration, indicating poor gastrointestinal absorption. Banerji *et al.* (1981) further demonstrated that purified seed saponins did not produce significant cardiovascular effects in dogs or haemolysis of human erythrocytes at therapeutic concentrations.

Traditional literature indicates that excessive consumption of fermented Mahua liquor may produce gastric irritation, nausea, and intoxication because of ethanol formation during fermentation (Nadkarni, 1954). Consequently, fermented flower products should be consumed responsibly and under appropriate regulatory guidelines.

Mahua seed cake contains substantial quantities of saponins that limit its direct use as animal feed. However, detoxification significantly reduces toxicity, and studies involving lactating buffaloes demonstrated no adverse effects on erythrocyte count, haemoglobin concentration, serum proteins, or general health following incorporation of detoxified Mahua seed cake into the diet (Pyne *et al.*, 1981). Similarly, detoxified seed cake is increasingly utilized as an organic fertilizer and biofertilizer without significant environmental hazards.

Recent investigations have also evaluated the genotoxicity and cytotoxicity of Mahua extracts and generally report low toxicity at therapeutic concentrations. Nevertheless, comprehensive chronic toxicity, reproductive toxicity, mutagenicity, carcinogenicity, pharmacokinetic, and human clinical safety studies remain insufficient. Future investigations should establish standardized dosage regimens, herb–drug interactions, and long-term safety profiles to facilitate regulatory approval of Mahua-derived phytopharmaceuticals.

Overall, current evidence suggests that *M. longifolia* is relatively safe when consumed traditionally or administered within recommended therapeutic doses. However, standardized processing, quality control, and rigorous toxicological evaluation are essential before its widespread pharmaceutical application.

BIOTECHNOLOGICAL APPLICATIONS

The increasing demand for sustainable natural products has stimulated considerable interest in the biotechnological utilization of *Madhuca longifolia* (J.F. Gmel.) J.F. Macbr. Advances in plant biotechnology, tissue culture, metabolomics, genetic improvement, and industrial biotechnology have expanded the commercial and pharmaceutical value of this multipurpose tree. Modern research focuses not only on conservation of elite germplasm but also on enhancing the production of bioactive compounds, improving propagation efficiency, and developing value-added products for food, pharmaceutical, cosmetic, and bioenergy industries (Patel & Naik, 2010; Grover *et al.*, 2022).

Micropropagation and In Vitro Regeneration

Conventional propagation of *M. longifolia* through seeds is limited by prolonged juvenile periods, irregular germination, and considerable genetic

variability. Consequently, micropropagation techniques have been developed for rapid multiplication of elite genotypes. Successful regeneration has been achieved using nodal segments, shoot tips, cotyledonary nodes, and immature embryos cultured on Murashige and Skoog (MS) medium supplemented with cytokinins such as benzylaminopurine (BAP), kinetin (Kn), and thidiazuron (TDZ). These protocols facilitate large-scale clonal propagation of disease-free planting material for afforestation, agroforestry, and commercial plantations (Rai *et al.*, 2016; Singh *et al.*, 2023).

Germplasm Conservation

Rapid deforestation, habitat degradation, and unsustainable harvesting have increased the need for conservation of *M. longifolia* genetic resources. Ex situ conservation through seed banks, field gene banks, cryopreservation, and in vitro repositories has become an important strategy for preserving elite germplasm. Tissue culture techniques further support long-term conservation and facilitate the exchange of disease-free planting material among research institutions (FAO, 2014; Grover *et al.*, 2022).

Metabolic Engineering and Secondary Metabolite Production

Recent advances in plant biotechnology have enabled investigations into the biosynthetic pathways responsible for the production of triterpenoids, flavonoids, phenolic compounds, and saponins in *M. longifolia*. Cell suspension cultures, callus cultures, hairy root cultures, and elicitor-based approaches are being explored to enhance the production of pharmacologically important secondary metabolites. Omics technologies, including genomics, transcriptomics, proteomics, and metabolomics, are expected to accelerate metabolic engineering for large-scale production of valuable phytochemicals (Grover *et al.*, 2022; Singh *et al.*, 2023).

Biodiesel and Bioenergy Production

Mahua seed oil has emerged as one of the most promising non-edible feedstocks for biodiesel production because of its high oil content (35–50%), favorable fatty acid composition, and widespread availability. Transesterification of Mahua oil yields biodiesel with physicochemical properties comparable to conventional diesel, making it a sustainable renewable energy source. Mahua-based biodiesel exhibits good cetane number, oxidative stability, biodegradability, and reduced greenhouse gas emissions, thereby contributing to rural energy security and environmental sustainability (Patel & Naik, 2010; Atabani *et al.*, 2013).

Fermentation and Industrial Biotechnology

The carbohydrate-rich flowers of *M. longifolia* are extensively utilized as substrates for microbial fermentation to produce ethanol, bioethanol, vinegar, wine, organic acids, enzymes, and probiotics. Owing to their high fermentable sugar content, Mahua flowers are increasingly recognized as valuable renewable biomass for biofuel industries and sustainable biorefineries. Modern fermentation technologies have significantly improved ethanol yield and product quality while minimizing waste generation (Patel & Naik, 2010; Singh *et al.*, 2023).

Nanobiotechnology

Recent studies have demonstrated the potential of Mahua extracts in green synthesis of metallic nanoparticles such as silver and zinc oxide nanoparticles. These phytosynthesized nanoparticles exhibit enhanced antimicrobial, antioxidant, wound-healing, and anticancer activities, opening new opportunities for biomedical applications. Green nanotechnology offers an eco-friendly alternative to conventional chemical synthesis and aligns with sustainable pharmaceutical development (Grover *et al.*, 2022).

Overall, biotechnology provides powerful tools for conservation, genetic improvement, metabolite enhancement, and industrial utilization of *Madhuca longifolia*, thereby supporting its sustainable commercialization and long-term utilization.

Pharmaceutical Applications

Madhuca longifolia has attracted considerable attention as a valuable source of bioactive phytochemicals with significant pharmaceutical potential. The presence of triterpenoids, flavonoids, phenolic acids, saponins, phytosterols, glycosides, and fatty acids has encouraged extensive investigations into the development of herbal medicines, phytopharmaceuticals, nutraceuticals, cosmeceuticals, and novel drug-delivery systems (Khare, 2007; Grover *et al.*, 2022).

Herbal Medicines

Extracts obtained from flowers, bark, leaves, and seeds are incorporated into traditional herbal formulations used for treating diabetes, inflammation, arthritis, skin diseases, respiratory disorders, gastrointestinal ailments, wounds, ulcers, and liver diseases. Modern pharmacological evidence validating antioxidant, antimicrobial, anti-inflammatory, antidiabetic, hepatoprotective, gastroprotective, and wound-healing properties supports the continued development of standardized herbal medicines derived from *M. longifolia* (Singh *et al.*, 2023).

Nutraceuticals and Functional Foods

The flowers are rich in carbohydrates, minerals, vitamins, amino acids, flavonoids, and phenolic antioxidants, making them excellent candidates for functional foods and nutraceutical products. Mahua flowers are increasingly utilized in the preparation of herbal beverages, syrups, jams, candies, bakery products, dietary supplements, fermented health drinks, and antioxidant-rich food formulations that promote nutritional security and preventive healthcare (Patel & Naik, 2010).

Cosmeceutical Applications

Mahua seed oil, commonly known as Mahua butter, possesses excellent emollient, moisturizing, anti-

inflammatory, and skin-conditioning properties. Consequently, it is extensively used in soaps, creams, lotions, lip balms, massage oils, shampoos, conditioners, sunscreens, and anti-aging formulations. The presence of essential fatty acids and natural antioxidants improves skin hydration, enhances wound healing, and protects against oxidative skin damage (Ramadan *et al.*, 2006; Singh *et al.*, 2023).

Drug Delivery Systems

Natural polysaccharides, seed oil, and mucilaginous components obtained from *M. longifolia* are being investigated as pharmaceutical excipients for controlled-release tablets, topical ointments, nanoemulsions, microemulsions, lipid nanoparticles, and transdermal drug-delivery systems. Mahua oil has shown promise as a biodegradable carrier for lipophilic drugs because of its biocompatibility and stability (Grover *et al.*, 2022).

Antimicrobial and Wound-Healing Formulations

Because of their broad-spectrum antimicrobial and wound-healing properties, Mahua extracts are increasingly incorporated into herbal antiseptic creams, gels, hydrogel dressings, ointments, and wound-care products. Their ability to reduce microbial growth, accelerate collagen synthesis, and promote tissue regeneration makes them attractive candidates for pharmaceutical wound management (Singh *et al.*, 2023).

Reproductive Health and Spermicidal Products

Triterpenoid saponins isolated from Mahua seeds have demonstrated potent spermicidal activity and have been investigated as potential plant-based contraceptive agents. These naturally occurring compounds offer prospects for developing safer, biodegradable, and affordable spermicidal formulations after further toxicological and clinical evaluation (Setty *et al.*, 1976; Banerji *et al.*, 1979).

Future Pharmaceutical Prospects

Recent advances in phytochemistry, molecular pharmacology, metabolomics, artificial intelligence-assisted drug discovery, and nanotechnology have accelerated the identification of novel therapeutic leads from *M. longifolia*. Bioactive constituents such as betulinic acid, lupeol, oleanolic acid, myricetin, quercetin, and triterpenoid saponins exhibit promising activities against chronic inflammatory diseases, diabetes, microbial infections, neurodegenerative disorders, cardiovascular diseases, and cancer. Future multidisciplinary studies involving pharmacokinetics, molecular docking, clinical trials, and regulatory standardization are expected to facilitate the development of evidence-based phytopharmaceuticals from this important medicinal tree (Grover *et al.*, 2022; Singh *et al.*, 2023).

Collectively, the available evidence indicates that *Madhuca longifolia* possesses enormous potential for the development of modern pharmaceuticals, nutraceuticals, cosmeceuticals, and sustainable bioproducts. Continued research integrating traditional knowledge with advanced biotechnology and pharmaceutical sciences will significantly enhance its commercial and therapeutic utilization.

CLINICAL STUDIES

Despite the extensive traditional use and encouraging pharmacological evidence, clinical investigations on *Madhuca longifolia* remain limited. Most available data originate from in vitro experiments, animal models, and ethnopharmacological observations. Well-designed randomized controlled clinical trials evaluating standardized Mahua preparations are still scarce. Consequently, current therapeutic applications are largely supported by traditional knowledge and preclinical evidence rather than high-quality clinical validation (Grover *et al.*, 2022; Singh *et al.*, 2023).

Traditional Clinical Use

For centuries, Mahua has been used by Ayurvedic physicians, Siddha practitioners, and tribal healers for managing diabetes, bronchitis, cough, asthma, skin diseases, rheumatism, wounds, ulcers, gastrointestinal disorders, malnutrition, reproductive disorders, and general weakness. Although these uses have been continuously practiced in Indian traditional medicine, systematic clinical documentation remains limited (Nadkarni, 1954; Khare, 2007).

Clinical Evidence in Diabetes

Experimental studies have demonstrated significant hypoglycemic and antioxidant activities of bark, flower, and leaf extracts in diabetic animal models. However, only a few preliminary observational studies and small pilot investigations have evaluated Mahua-based herbal formulations in patients with type 2 diabetes. These studies suggest improvements in fasting blood glucose and oxidative stress markers when Mahua is administered as part of polyherbal Ayurvedic formulations. Nevertheless, the individual therapeutic contribution of *M. longifolia* cannot yet be conclusively established because of limited sample sizes and lack of placebo-controlled clinical trials (Grover *et al.*, 2022).

Clinical Applications in Wound Healing and Dermatology

Traditional topical preparations containing Mahua seed oil, bark, and leaf extracts are widely used in rural India for treating chronic wounds, burns, eczema, cracked skin, ulcers, and inflammatory skin disorders. Clinical observations indicate accelerated wound healing and improved tissue regeneration, although these reports are largely anecdotal or derived from traditional medical practice. Controlled clinical trials evaluating standardized topical formulations are still lacking (Khare, 2007; Singh *et al.*, 2023).

Nutritional and Functional Food Applications

Mahua flowers have long been consumed as nutrient-rich traditional foods and beverages by tribal communities. Their high carbohydrate, mineral, vitamin, and antioxidant contents have encouraged their development as functional foods and nutraceuticals. Community-based nutritional studies suggest that Mahua flowers contribute to food security and provide supplementary dietary energy during periods of seasonal food scarcity. However, rigorous human intervention studies evaluating their effects on nutritional status, antioxidant capacity, metabolic syndrome, or chronic diseases remain unavailable (Patel & Naik, 2010).

Reproductive Health

Among the various pharmacological properties of *M. longifolia*, the spermicidal activity of seed saponins has received considerable scientific attention. Laboratory investigations have demonstrated effective spermicidal action in human semen in vitro, suggesting the possibility of developing plant-derived contraceptive formulations (Setty *et al.*, 1976; Banerji *et al.*, 1979). However, no approved clinical contraceptive products based on Mahua saponins are currently available, and comprehensive human clinical trials evaluating efficacy and safety have not yet been completed.

Safety in Humans

Traditional dietary consumption of Mahua flowers and refined seed oil indicates a generally favorable safety profile. Available toxicological evidence suggests that properly processed plant materials are safe when consumed in recommended quantities. Nevertheless, excessive intake of fermented Mahua liquor may result in ethanol-related adverse effects, while raw seed saponins require detoxification before use because of their potential toxicity. To date, no comprehensive Phase I–III clinical trials have established standardized therapeutic doses, long-term safety, pharmacokinetics, or herb–drug interactions of Mahua-derived preparations (Mulky & Gandhi, 1977; Banerji *et al.*, 1981).

Future Clinical Perspectives

Given the promising pharmacological profile of *Madhuca longifolia* (J.F. Gmel.) J.F. Macbr., there is an urgent need to translate the growing body of preclinical evidence into well-designed human clinical studies. Future research should prioritize randomized, double-blind, placebo-controlled clinical trials to establish the efficacy, optimal dosage, and therapeutic safety of standardized Mahua formulations. Standardization of plant extracts using validated phytochemical markers and advanced analytical techniques such as HPLC, LC–MS/MS, and metabolomic fingerprinting is essential to ensure batch-to-batch consistency and reproducibility. Comprehensive pharmacokinetic, pharmacodynamic, and bioavailability studies are required to elucidate the absorption, distribution, metabolism, excretion, and mechanisms of action of its bioactive constituents. In addition, long-term toxicological investigations, including chronic toxicity, reproductive toxicity, genotoxicity, carcinogenicity, and herb–drug interaction studies, are necessary to establish an evidence-based safety profile. Clinical evaluations should focus on diseases for which substantial preclinical evidence exists, including type 2 diabetes mellitus, inflammatory and autoimmune disorders, wound healing, liver diseases, metabolic syndrome, cardiovascular diseases, microbial infections, dermatological disorders, and selected cancers. Furthermore, Mahua-derived nutraceuticals, phytopharmaceuticals, cosmeceuticals, and botanical drug formulations should be developed and evaluated according to internationally accepted regulatory frameworks, including WHO, ICMR, AYUSH, USFDA, and EMA guidelines. The integration of omics technologies, artificial intelligence-assisted drug discovery, systems pharmacology, and precision medicine approaches may further accelerate the identification of novel therapeutic candidates from *M. longifolia*. Such multidisciplinary investigations will provide robust scientific validation of its traditional medicinal uses, facilitate regulatory approval, and promote the integration of Mahua-based products into evidence-based modern healthcare. Although current preclinical findings strongly support the therapeutic potential of *Madhuca longifolia*, its successful translation into clinical practice ultimately depends on rigorous human clinical research and the development of standardized, safe, and effective pharmaceutical formulations.

CONCLUSION

Madhuca longifolia (J.F. Gmel.) J.F. Macbr. (Mahua) is one of the most valuable indigenous multipurpose tree species of the Indian subcontinent, possessing exceptional ethnomedicinal, nutritional, ecological, and socioeconomic importance. For centuries, it has occupied a prominent position in Ayurveda, Siddha, Unani, and various indigenous healthcare systems, where its flowers, leaves, bark, fruits, seeds, and seed oil have been extensively utilized for the treatment of diabetes, inflammatory disorders, respiratory diseases, gastrointestinal ailments, skin infections, wound healing, liver disorders, reproductive problems, and general debility. The continued reliance of tribal communities on Mahua further highlights its indispensable role in traditional healthcare, food security, and rural livelihoods.

Phytochemical investigations conducted over the past six decades have revealed that *M. longifolia* is an abundant source of biologically active constituents, including triterpenoids, triterpenoid saponins, flavonoids, phenolic compounds, phytosterols, glycosides, fatty acids, tannins, vitamins, and essential minerals. These compounds are responsible

for its broad spectrum of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, antidiabetic, hepatoprotective, gastroprotective, wound-healing, analgesic, immunomodulatory, antiviral, spermicidal, and anticancer effects. Modern analytical techniques such as HPLC, LC–MS/MS, GC–MS, metabolomics, and molecular pharmacology have substantially improved our understanding of its phytochemical diversity and therapeutic mechanisms.

In addition to its medicinal significance, Mahua is recognized as an important nutraceutical and industrial crop. Its carbohydrate-rich flowers serve as valuable nutritional resources and raw materials for fermented beverages, bioethanol, confectionery products, and functional foods, whereas its oil-rich seeds provide renewable feedstock for edible fats, cosmetics, pharmaceuticals, soaps, lubricants, and biodiesel production. Recent advances in biotechnology, including tissue culture, germplasm conservation, metabolic engineering, nanobiotechnology, and phytopharmaceutical development, have further expanded the commercial potential of this species.

Although numerous experimental studies have validated many traditional medicinal claims, high-quality clinical evidence remains limited. Most pharmacological investigations have been confined to in vitro and animal studies, with relatively few well-designed human clinical trials. Therefore, future research should prioritize standardized extract development, identification of bioactive marker compounds, pharmacokinetic investigations, toxicological evaluation, molecular mechanism studies, randomized controlled clinical trials, and evidence-based formulation development. Integration of modern omics technologies, artificial intelligence-assisted drug discovery, systems pharmacology, and precision medicine approaches may accelerate the identification of novel therapeutic agents from *Madhuca longifolia*.

Furthermore, increasing anthropogenic pressures, habitat degradation, overexploitation, and climate change necessitate the implementation of comprehensive conservation and sustainable utilization strategies. Large-scale cultivation, ex situ and in situ conservation, community-based resource management, and value-addition initiatives will not only conserve this ecologically important species but also enhance the livelihoods of forest-dependent and tribal communities.

In conclusion, *Madhuca longifolia* represents a remarkable example of a traditional medicinal tree whose historical importance is increasingly supported by contemporary scientific evidence. Its unique combination of nutritional value, diverse phytochemistry, wide-ranging pharmacological activities, industrial applications, and biotechnological potential makes it a highly promising candidate for the development of future phytopharmaceuticals, nutraceuticals, cosmeceuticals, and sustainable bio-based products. Continued multidisciplinary research integrating ethnobotany, phytochemistry, pharmacology, biotechnology, clinical medicine, and conservation biology will be essential for fully realizing the therapeutic and commercial potential of this "Tree of Life" while ensuring its sustainable utilization for future generations.

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RESEARCH ARTICLE

OPTIMIZING CALLUS CULTURE FOR HIGH-PEROXIDASE PRODUCTION: A CASE STUDY OF *VITEX* WITH UNIQUE ENZYME CHARACTERISTICS

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Abstract: Efficient callus induction and proliferation protocols have been successfully established for several plant species known to be potent sources of peroxidases. Phenolic compounds, while inhibitory to callus formation, have been found to stimulate peroxidase production. The level of peroxidase activity in callus cultures varies significantly depending on both the type of plant growth regulator used and the morphological nature of the callus. Among the species studied, *Vitex* peroxidase is particularly unique, as callus cultures exhibit exceptionally high enzyme activity despite minimal or undetectable activity in the intact plant body. N-terminal sequencing of the *Vitex* peroxidase revealed a novel sequence distinct from all previously reported plant peroxidases, which likely accounts for its remarkably high specific activity. These findings highlight the potential of *Vitex* callus cultures as a valuable biotechnological system for peroxidase production.

Keywords: Callus culture, Peroxidase, *Vitex*, N-terminal sequencing

INTRODUCTION

Peroxidases (EC 1.11.1.x) are heme-containing oxidoreductase enzymes that catalyze the oxidation of a wide variety of organic and inorganic substrates using hydrogen peroxide as an electron acceptor. These enzymes play crucial roles in plant growth, lignification, defense responses, wound healing, and stress adaptation. Owing to their broad substrate specificity and catalytic efficiency, peroxidases have gained significant industrial importance in biotechnology, clinical diagnostics, biosensor development, wastewater treatment, bioremediation, food processing, and pharmaceutical applications. Recent studies have highlighted the expanding physiological and biotechnological significance of Class III plant peroxidases, emphasizing the need for novel sources of highly active and stable enzymes (Freitas et al., 2024).

Commercial production of peroxidases is largely dependent on horseradish peroxidase (HRP), which remains the most widely used plant-derived enzyme. However, increasing industrial demand, coupled with the need for enzymes possessing enhanced catalytic activity, stability, and cost-effective production systems, has stimulated the search for alternative plant sources. Plant tissue culture technology offers an attractive platform for enzyme production because it enables controlled growth conditions, independence from seasonal variations, and the possibility of enhancing metabolite and enzyme yields through manipulation of culture conditions. *In vitro* cultures, particularly callus and suspension cultures, often exhibit altered gene expression patterns that can lead to the synthesis of valuable biomolecules absent or minimally expressed in intact plants.

Vitex negundo Linn. (Nirgundi) is an important medicinal shrub widely distributed throughout India and other parts of Asia. The plant has been extensively utilized in traditional medicine systems including Ayurveda, Siddha, and Unani for the treatment of inflammatory disorders, respiratory diseases, microbial infections, pain, and various chronic ailments. Modern investigations have revealed that *V. negundo* contains diverse bioactive constituents, including flavonoids, iridoids, lignans, phenolic acids, and terpenoids that contribute to its broad pharmacological activities. Recent reviews have further documented its antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, neuroprotective, and anticancer potential, highlighting its growing importance as a medicinal and biotechnological resource (Dube *et al*., 2024; Review, 2026).

Although considerable attention has been directed toward the phytochemical and pharmacological properties of *V. negundo*, relatively little information is available regarding its potential as a source of industrially important enzymes. An intriguing observation from preliminary investigations is that the intact plant exhibits negligible peroxidase activity, whereas its callus cultures produce substantial quantities of the enzyme. Such

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differential expression may result from activation of stress-responsive genes and metabolic pathways during cellular dedifferentiation and callus formation. The phenomenon provides a unique opportunity to investigate the regulation of peroxidase biosynthesis while simultaneously developing an alternative source of commercially valuable enzymes.

The present study was therefore undertaken to establish efficient protocols for callus induction, proliferation, and maintenance in *Vitex negundo* and to evaluate the influence of culture conditions on peroxidase production. Furthermore, the extracellular peroxidase produced by the callus cultures was purified and characterized with respect to its biochemical properties, molecular weight, optimum reaction conditions, and N-terminal amino acid sequence. The study also aimed to compare the characteristics of the purified enzyme with commercially available horseradish peroxidase and to assess its novelty and potential industrial applicability. The findings provide valuable insights into the unique expression of peroxidase in *V. negundo* callus cultures and support the development of plant tissue culture-based systems for sustainable enzyme production.

MATERIALS AND METHODS

Plant Material and Explant Preparation

Healthy plants of *Vitex negundo* Linn. were collected from the vicinity of the Regional Research Laboratory (RRL), Thiruvananthapuram, India. Young leaves were selected as explants because of their high regenerative potential and suitability for callus induction. The explants were washed thoroughly under running tap water, followed by surface sterilization using standard aseptic procedures employed in plant tissue culture studies. Recent advances in medicinal plant biotechnology have demonstrated that leaf explants are highly responsive for in vitro regeneration and metabolite production.

Callus Induction and Proliferation

Surface-sterilized leaf explants were cultured on Murashige and Skoog (MS) basal medium supplemented with different combinations of 6-benzylaminopurine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D). The cultures were incubated at 25 ± 2°C under a 16 h photoperiod. Callus induction was assessed after four weeks of culture.

The optimum callus induction response was obtained on MS medium containing 0.5 mg L^{-1} 2,4-D and 0.3 mg L^{-1} BA. The induced callus was compact and yellow in appearance. Similar cytokinin–auxin interactions have been reported to regulate cellular dedifferentiation and biomass production in medicinal plant cultures (Kruglova et al., 2023).

Establishment of Cell Suspension Cultures

Compact callus aggregates (CCAs) obtained during subculturing were transferred to liquid MS medium and maintained on a rotary shaker at 100–120 rpm. Suspension cultures were periodically sub cultured to ensure continuous proliferation and extracellular enzyme production. Plant cell suspension cultures have been recognized as effective systems for the production of high-value enzymes and secondary metabolites because of their scalability and controlled growth conditions.

Regeneration Studies

To investigate the effect of organogenesis on peroxidase production, callus cultures were transferred to regeneration medium containing varying concentrations of coconut water (CW). Among the concentrations tested, 1% (v/v) CW produced the highest regeneration frequency. Morphogenic responses were recorded at different stages, and corresponding peroxidase activities were measured. Coconut water is known to contain cytokinin-like compounds such as zeatin and has been widely used to promote shoot organogenesis in plant tissue culture systems.

Extraction and Peroxidase Assay

Extracellular peroxidase was harvested from suspension culture media by filtration through sterile cheesecloth to remove cellular debris. Enzyme activity was determined spectrophotometrically using ABTS, guaiacol, and o-phenylenediamine (o-PDA) as substrates in the presence of hydrogen peroxide. Peroxidase assays were performed according to standard enzymatic procedures routinely employed for plant peroxidase characterization (Freitas *et al.*, 2024).

One unit of enzyme activity was defined as the amount of enzyme required to catalyze the oxidation of 1 µmol of substrate per minute under assay conditions.

Purification of Peroxidase

The crude enzyme preparation was concentrated using ultrafiltration membranes with a molecular weight cut-off of 10 kDa. The concentrated sample was subsequently lyophilized and subjected to preparative isoelectric focusing using a Rotofor apparatus. Fractions exhibiting maximum enzyme activity were collected and used for further characterization.

Purification strategies involving ultrafiltration and isoelectric focusing are commonly employed for obtaining highly active plant peroxidases suitable for biochemical characterization (Freitas et al., 2024).

SDS–PAGE Analysis

The purified enzyme was analyzed by SDS–PAGE using a 12% resolving gel and a 5% stacking gel. Electrophoresis was carried out at 200 V and 110 mA, followed by Coomassie Brilliant Blue staining. Molecular weight determination was performed using standard protein markers. SDS–PAGE remains

the most widely adopted technique for evaluating purity and molecular mass of plant-derived enzymes.

Determination of Optimum pH and Temperature

The effect of pH on enzyme activity was investigated using appropriate buffer systems over a broad pH range. Similarly, enzyme activity was measured at different temperatures to determine the optimum reaction temperature. The pH and temperature profiles were constructed from relative enzyme activity values.

Effect of Metal Ions on Enzyme Activity

The influence of selected metal ions and inhibitors on enzyme activity was studied by incubating the purified enzyme with 1 mM concentrations of KI, NaN_3, $MgSO_4$, $ZnSO_4$, $MnSO_4$, $CaCl_2$, $HgCl_2$, and NaCl. Residual activity was measured under standard assay conditions and expressed as a percentage of the untreated control.

Protein Estimation

Protein concentration was determined using bovine serum albumin (BSA) as the standard. Specific activity was calculated as enzyme units per milligram of protein.

N-Terminal Amino Acid Sequencing and Bioinformatic Analysis

Purified peroxidase was electrophoresed on SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The protein band corresponding to the purified enzyme was excised and subjected to automated Edman degradation using an Applied Biosystems 477A Protein Sequencer.

The obtained amino acid sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) to identify homologous proteins in public databases. Sequence comparison and molecular characterization are widely employed to establish enzyme novelty and evolutionary relationships among plant peroxidases (Freitas et al., 2024).

Statistical Analysis

All experiments were conducted in triplicate and the results were expressed as mean ± standard deviation. Statistical significance among treatments was determined using one-way analysis of variance (ANOVA), and differences were considered significant at $p < 0.05$.

RESULTS

Callus Induction and Proliferation

Leaf explants of *Vitex negundo* responded successfully to in vitro culture conditions and produced callus on Murashige and Skoog (MS) medium supplemented with different combinations of plant growth regulators. Among the treatments tested, MS medium containing 0.5 mg L^{-1} 2,4-D and 0.3 mg L^{-1} BA produced the highest callus induction frequency. The induced callus was compact, yellowish, and actively proliferating.

Subsequent subculturing of the callus on medium containing 1.0 mg L^{-1} BA resulted in the formation of compact callus aggregates (CCAs). These aggregates exhibited rapid growth and maintained their structural integrity during repeated subcultures. Both compact and friable callus forms were obtained depending on the hormonal composition of the culture medium.

Effect of Growth Regulators on Peroxidase Production

Peroxidase production varied considerably with the type and concentration of growth regulators used. Calli cultured in hormone-free medium exhibited relatively high peroxidase activity but showed poor biomass accumulation. Auxins such as IAA, NAA, and 2,4-D generally reduced enzyme production and callus growth when supplied individually.

In contrast, cytokinin supplementation with BA significantly enhanced both biomass production and enzyme activity. The combination of 1.5 mg L^{-1} BA and 1.0 mg L^{-1} 2,4-D was found to be optimal for simultaneous biomass accumulation and peroxidase production.

Regeneration and Peroxidase Activity

The addition of coconut water (CW) induced organogenesis in the non-organogenic callus cultures. Among the concentrations tested, 1% (v/v) CW produced the highest regeneration response. Progressive development of shoots was observed during the regeneration process.

Analysis of peroxidase activity during different stages of regeneration revealed a gradual decline in enzyme production with increasing differentiation of the callus tissue. The highest enzyme activity was recorded in undifferentiated callus cultures, whereas regenerated tissues exhibited substantially lower activity levels.

Growth Kinetics and Enzyme Production

The suspension cultures of *V. negundo* demonstrated active biomass accumulation throughout the cultivation period. Peroxidase production increased concomitantly with cell growth and reached maximum levels during the late exponential phase of culture growth. Thereafter, enzyme production stabilized with only minor fluctuations.

The relationship between biomass accumulation and enzyme production indicated that peroxidase synthesis was closely associated with active cellular proliferation (Fig 1).

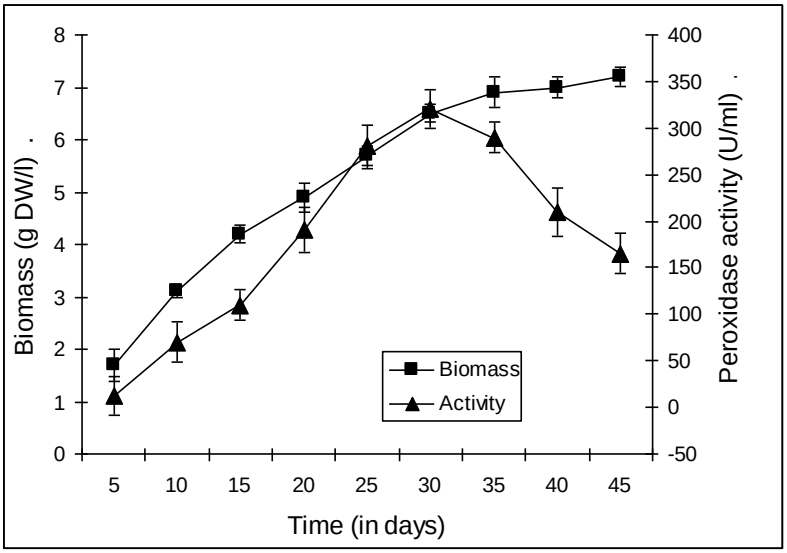

Fig 1: Impact of time course on peroxidase activity and biomass growth of *Vitex*

Purification of Extracellular Peroxidase

The extracellular peroxidase produced by suspension cultures was successfully purified through ultrafiltration, lyophilization, and preparative isoelectric focusing. Multiple isozyme fractions were detected; however, the major active fraction was focused at an isoelectric point (pI) of approximately 9.0.

Purification resulted in a substantial increase in enzyme specific activity. The purified enzyme exhibited nearly nine-fold enrichment compared with the crude extract, indicating the effectiveness of the purification procedure.

Molecular Weight Determination

SDS–PAGE analysis of the purified enzyme revealed a single prominent protein band, indicating a high degree of purity. The molecular mass of the purified *Vitex* peroxidase was estimated to be approximately 34 kDa (Figure 2).

This molecular weight was lower than that reported for commercial horseradish peroxidase, which typically exhibits a molecular mass of approximately 40 kDa.

Biochemical Characterization of Purified Peroxidase

Optimum Temperature

The purified peroxidase exhibited maximum catalytic activity at 45°C. Enzyme activity gradually declined at temperatures above the optimum, indicating thermal sensitivity of the protein structure (Fig 2)

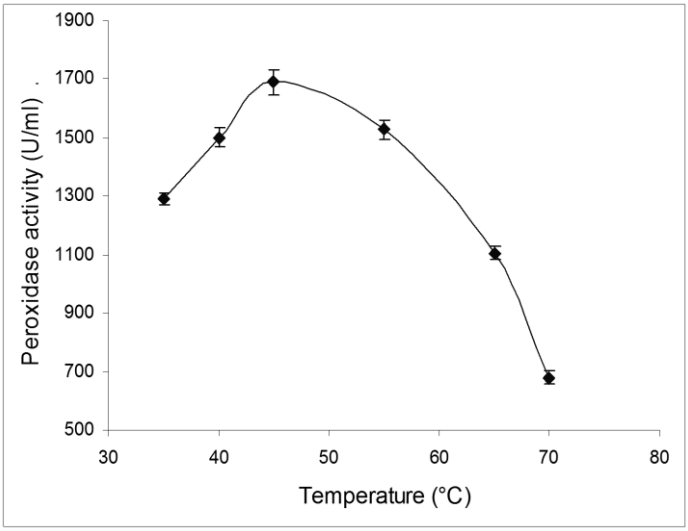

Fig 2: Optimum temperature of purified *Vitex* peroxidase

Optimum pH

Maximum enzyme activity was observed at pH 5.5. Activity decreased significantly at both acidic and alkaline extremes, suggesting that the catalytic efficiency of the enzyme is strongly influenced by hydrogen ion concentration (Fig 3)

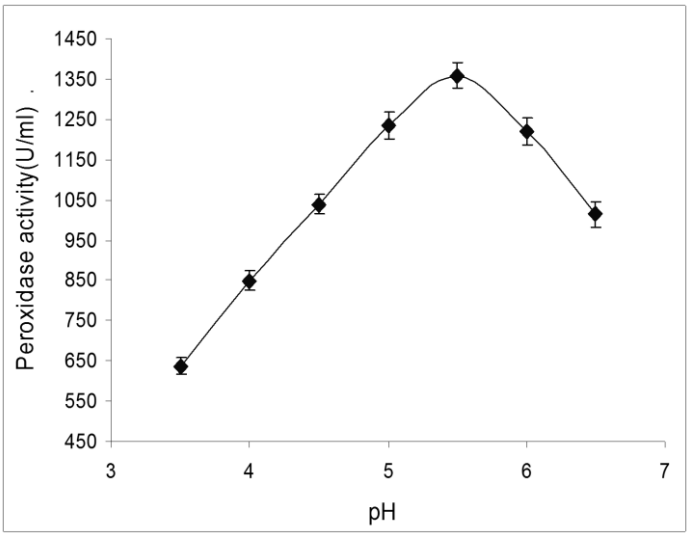

Fig 3: Optimum pH of purified *Vitex* peroxidase

Effect of Metal Ions and Chemical Reagents

The influence of selected metal ions and inhibitors on purified peroxidase activity is summarized in Table 1. $ZnSO_4$ produced the highest activation effect, increasing enzyme activity to 138% of the control value. $MnSO_4$ and $CaCl_2$ also enhanced activity, resulting in 131% and 130% relative activity, respectively.

Conversely, NaN_3 and $HgCl_2$ strongly inhibited enzyme activity, reducing activity to 73% and 62% of the control, respectively. KI slightly stimulated enzyme activity, whereas $MgSO_4$ and NaCl produced only marginal effects.

Table 1. Effect of metal ions on purified *Vitex negundo* peroxidase activity

Reagent (1 mM)	**Relative Activity (%)**
Control	100
KI	116
NaN_3	73
$MgSO_4$	97
$ZnSO_4$	138
$MnSO_4$	131
$CaCl_2$	130
$HgCl_2$	62
NaCl	103

Comparison with Commercial Horseradish Peroxidase

The crude extracellular enzyme preparation from *V. negundo* exhibited specific activities of 1572 U mg^{-1} protein using ABTS and 544 U mg^{-1} protein using guaiacol as substrates. Following purification, the specific activity increased to 14,730 U mg^{-1} protein using ABTS (Table 2).

Remarkably, even the crude enzyme preparation demonstrated activity levels comparable to those of commercial horseradish peroxidase (HRP). The purified enzyme showed substantially higher specific activity than the commercial reference enzyme, highlighting its potential industrial significance.

Table 2. Comparative chart of nirgundi peroxidase with known Horseradish peroxidase

Properties	**Horseradish peroxidase (Sigma)**	***V. negundo***	
		crude	pure
Molecular weight (kDa)	40	-	34
R.Z (Reinheitszahl) value	2.0		2.10
pH optimum	6–7	4.5	5.5
Optimum temperature (˚C)	50	50	45
Specific activity (Units/mg of protein):			
Using ABTS:			
Using Gaiacol:	1820	1572	14730 ND*
	520	544	

N-Terminal Amino Acid Sequence Analysis

The N-terminal amino acid sequence of the purified peroxidase was determined as:

ADIPYNGNGAV

BLAST analysis revealed very low sequence similarity with previously reported plant peroxidases. The highest similarity observed was approximately

18%, indicating that the enzyme possesses a unique amino acid sequence (Fig 4)

Sequence alignment with known peroxidases from horseradish, soybean, barley, wheat, tobacco, Arabidopsis, and other plant sources confirmed the distinct nature of the *Vitex* enzyme. The low homology strongly suggests that the enzyme represents a novel peroxidase isoform.

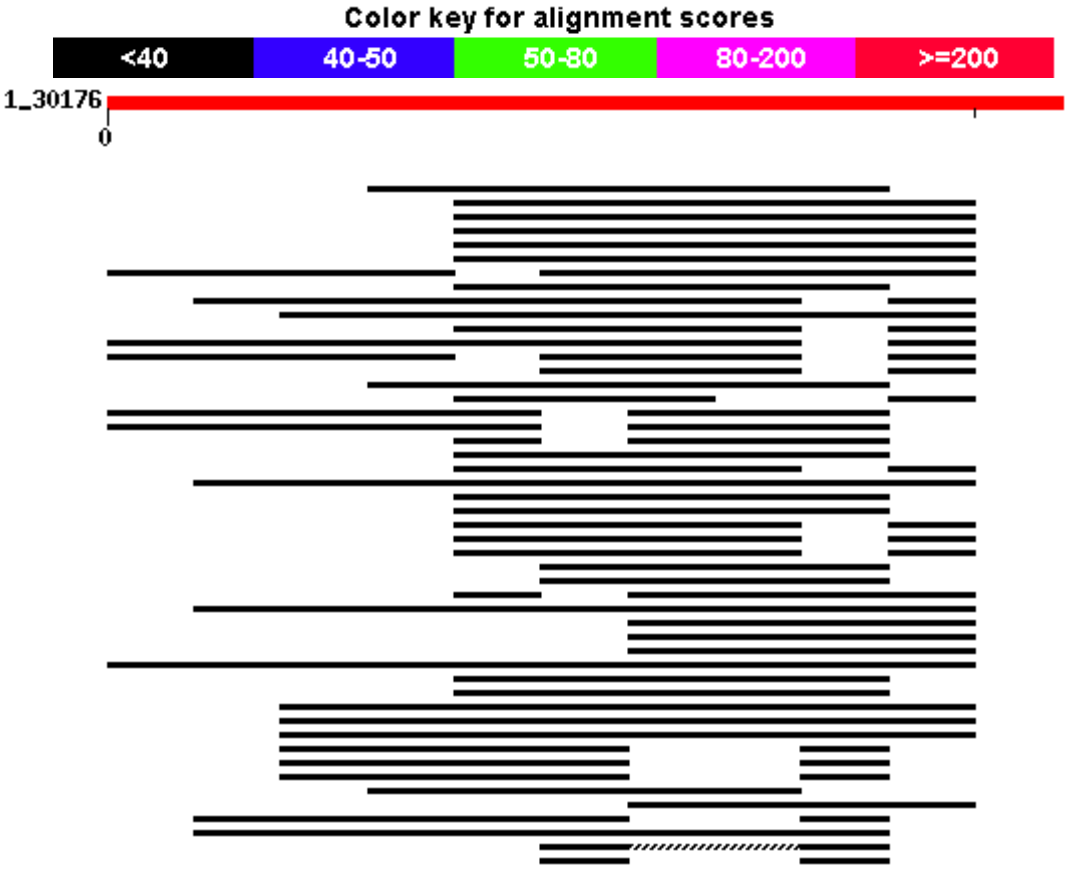

Fig 4: BLAST results of query amino acid sequence, ADIPYNGNGAV

Most Related Structures

Sequences producing significant alignments:	**Score**	(**bits**) Value
gi|55701055|tpe|CAH69336.1| TPA: class III peroxidase 94 pr...	18	12
gi|166807|gb|AAA32842.1| peroxidase	18	12
gi|16323436|gb|AAL15212.1| putative peroxidase [Arabidopsis...	18	12
gi|62319823|dbj|BAD93845.1| peroxidase like protein [Arabid...	18	12
gi|17530564|gb|AAL40849.1| class III peroxidase ATP34 [Arab...	18	12
gi|129812|sp|P17180|PER3_ARMRU Peroxidase C3 precursor	18	12
gi|26398007|sp|P59121|PERE5_ARMRU Peroxidase E5	18	12
gi|55700917|tpe|CAH69267.1| TPA: class III peroxidase 25 pr...	17	21
gi|4504183|ref|NP_000843.1| glutathione transferase [Homo s...	16	28
gi|13475896|ref|NP_107466.1| hypothetical protein mll7076 [...	16	37
gi|25285624|pir||B98301 non-heme chloroperoxidase (chloride...	16	37
gi|55700915|tpe|CAH69266.1| TPA: class III peroxidase 24 pr...	15	50
gi|1781334|emb|CAA71494.1| peroxidase [Spinacia oleracea]	15	67
gi|60687493|gb|AAX35769.1| fatty acid oxygenase [Emericella...	15	67
gi|17135659|dbj|BAB78205.1| all7121 [Nostoc sp. PCC 7120] >...	15	90
gi|55700883|tpe|CAH69251.1| TPA: class III peroxidase 8 pre...	14	121
gi|24196840|gb|AAN50172.1| Methylamine utilization protein ...	14	121
gi|37962659|gb|AAR05646.1| alpha-DOX1 [Lycopersicon esculen...	14	121
gi|55700957|tpe|CAH69287.1| TPA: class III peroxidase 45 pr...	14	121

gi|444058|prf||1906181A	lignin peroxidase	14	121
gi|732970|emb|CAA59484.1|	pox1 [Triticum aestivum] >gi|2117...	14	163
gi|30793970|gb|AAP40436.1|	putative peroxidase [Arabidopsis...	14	163

The following Fig 5 shows a comparison of amino acid sequences of some of the reported peroxidases from selected sources.

AKPC	N	P	T	D	V	V	A	L	S	G	G	H	T
TOB	T	P	T	D	V	V	A	L	S	G	G	H	T
ATP	N	I	T	D	V	V	A	L	S	G	A	H	T
HRPC	R	S	S	D	L	V	A	L	S	G	G	H	T
SBP	N	T	L	D	L	V	T	L	S	G	G	H	T
BP1	D	A	T	D	L	V	T	I	S	G	G	H	T
WP	L	T	I	D	M	V	A	L	S	G	A	H	T
APX	S	D	Q	D	I	V	A	L	S	G	G	H	T
LiP 2	P	A	P	D	G	L	V	P	E	P	F	H	T
ARP	S	P	D	E	V	V	D	L	L	A	A	H	S

Fig 5: Amino acid sequence alignment of 13 amino acids sequence of a fragment for different peroxidases.

DISCUSSION

The present study demonstrated that callus cultures of *Vitex negundo* constitute an efficient source of extracellular peroxidase. A particularly noteworthy observation was the differential expression of peroxidase between the intact plant and its callus cultures. While the general plant tissues exhibited negligible enzyme activity, the callus cultures produced substantial amounts of peroxidase. Such differential expression is commonly associated with cellular dedifferentiation and stress-induced activation of metabolic pathways during in vitro culture. Recent studies have shown that plant tissue cultures frequently exhibit altered gene expression patterns and enhanced synthesis of stress-responsive enzymes compared with differentiated tissues (Freitas *et al.,* 2024).

The successful induction of callus using BA and 2,4-D confirms the importance of auxin–cytokinin interactions in regulating cellular proliferation and metabolic activity. Similar findings have been reported in several medicinal plants where optimized hormone combinations significantly enhanced biomass production and accumulation of valuable metabolites (Sharma *et al.,* 2023). The superior performance of compact callus aggregates observed in the present study may be attributed to improved cellular organization and physiological stability, which facilitate enhanced enzyme biosynthesis.

The gradual decline in peroxidase activity during regeneration suggests that enzyme production is associated primarily with the undifferentiated state of the tissue. Peroxidases are known to participate in stress signaling, cell wall remodeling, lignification, and reactive oxygen species (ROS) detoxification, processes that are generally more active in proliferating callus cells than in differentiated tissues (Li *et al.,* 2024). Similar reductions in enzyme activity during organogenesis have been reported in other plant tissue culture systems and are often linked to metabolic reprogramming accompanying cellular differentiation.

The growth-associated pattern of enzyme production observed in suspension cultures indicates that peroxidase synthesis is closely related to active cellular metabolism. Such findings are advantageous for industrial enzyme production because biomass accumulation and enzyme synthesis can be optimized simultaneously. Cell suspension cultures have been increasingly recognized as sustainable platforms for large-scale production of enzymes and secondary metabolites due to their scalability, controlled growth conditions, and year-round productivity.

Purification studies revealed a major extracellular peroxidase isoform with a molecular mass of approximately 34 kDa and an isoelectric point near pI 9.0. Plant Class III peroxidases typically exhibit molecular masses ranging from 30 to 45 kDa, depending on their amino acid composition and glycosylation patterns (Freitas *et al.,* 2024). The molecular weight observed for *V. negundo* peroxidase therefore falls within the expected range but differs from commercial horseradish peroxidase, which generally possesses a molecular mass of approximately 40 kDa. Such differences may reflect species-specific structural variations and post-translational modifications.

The purified enzyme displayed optimum catalytic activity at pH 5.5 and 45°C. These characteristics are consistent with the acidic pH preference commonly reported for plant peroxidases. Similar optimum conditions have been documented for peroxidases isolated from horseradish, soybean, tobacco, and

several other plant sources (Freitas *et al*., 2024). The ability of the enzyme to retain substantial activity at elevated temperatures further supports its potential utility in industrial applications requiring moderate thermal stability.

The effects of metal ions on enzyme activity provided additional insights into the biochemical characteristics of the purified enzyme. Zinc, manganese, and calcium ions enhanced catalytic activity, whereas sodium azide and mercury ions strongly inhibited the enzyme. Sodium azide is a well-known inhibitor of heme-containing peroxidases because it binds directly to the heme prosthetic group and interferes with electron transfer reactions. Similar inhibition patterns have been reported for other Class III plant peroxidases (Li *et al.*, 2024). The stimulatory effects of divalent cations may be associated with stabilization of enzyme conformation and enhancement of substrate binding efficiency.

One of the most significant findings of this investigation was the exceptionally high specific activity of the purified enzyme. Even the crude extracellular preparation exhibited activity comparable to commercial horseradish peroxidase, while purification resulted in an approximately nine-fold increase in specific activity. The growing demand for highly efficient peroxidases in biosensors, clinical diagnostics, wastewater treatment, and biocatalytic processes has stimulated the search for alternative enzyme sources (Freitas *et al.*, 2024). Therefore, the high catalytic efficiency exhibited by *V. negundo* peroxidase highlights its potential industrial significance.

The N-terminal amino acid sequence analysis revealed the sequence ADIPYNGNGAV, which exhibited only limited similarity with previously reported plant peroxidases. BLAST analysis showed a maximum sequence identity of approximately 18%, suggesting that the enzyme may represent a novel peroxidase isoform. Class III plant peroxidases constitute a highly diverse multigene family characterized by substantial sequence variation despite conservation of catalytic function (Li *et al.*, 2024). The low sequence homology observed in the present study, together with the unusually high specific activity of the enzyme, supports the hypothesis that *V. negundo* callus cultures express a structurally distinct peroxidase.

Although *Vitex negundo* has been extensively investigated for its medicinal properties, including antimicrobial, antioxidant, anti-inflammatory, hepatoprotective, and anticancer activities (Dube *et al.*, 2024), information regarding its industrial enzyme potential remains scarce. The present study therefore expands the biotechnological significance of this medicinal species by demonstrating that its callus cultures can serve as a sustainable source of a highly active and potentially novel peroxidase.

Overall, the results establish *Vitex negundo* callus cultures as a promising platform for peroxidase production. The unique expression pattern, high catalytic activity, favorable biochemical properties, and distinctive N-terminal sequence collectively suggest considerable potential for industrial and biotechnological applications. Future studies involving complete gene sequencing, recombinant expression, kinetic characterization, and structural analysis will be valuable for further understanding the molecular basis of the enzyme's exceptional activity and novelty.

CONCLUSION

Very effective protocol could be established for the callus induction and proliferation of some selected plants that are potent sources of peroxidases. Phenolic substances were found to increase peroxidase production, though it inhibited callus formation. The calluses showed differences in peroxidase production depending on the type of growth regulator used and also the nature of callus. *Vitex* peroxidase was very unique as the callus cultures expressed tremendous enzyme activity even though the general plant body lacked it. The N-terminal sequencing of the *Vitex* peroxidase was found to be different from the other reported sequences and that may be accounting for the high specific activity of the enzyme.

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RESEARCH ARTICLE

BHANG ESSENTIAL OIL POSSESSES ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY

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Abstract: The present study evaluated the antioxidant and antibacterial activity of essential oil extracted from fresh leaves of *Cannabis sativa.* Antioxidant potential was determined using DPPH radical scavenging, ferric reducing antioxidant power (FRAP) assay, while antibacterial efficacy was assessed by the agar well diffusion method against gram-positive and gram-negative bacterial strains. The essential oil of the plant exhibited significant antioxidant potential, as evidenced by its free radical scavenging ability and ferric ion reducing capacity and also inhibited the growth of all tested bacterial strains, with the highest antibacterial activity observed against *Escherichia coli*, followed by *Micrococcus luteus.* The findings indicate that *C. sativa* essential oil possesses promising antioxidant and antibacterial properties, which may be attributed to the synergistic action of its volatile phytoconstituents. The results highlight its potential for future applications as a natural bioactive ingredient in pharmaceutical, nutraceutical and food preservation systems.

Keywords: *Cannabis sativa*, Essential oil, DPPH, FRAP, Antibacterial

INTRODUCTION

Cannabis sativa, commonly known as bhang or Indian hemp, is an annual herbaceous plant belonging to the family Cannabaceae, native to Central and South Asia and widely used in traditional medicine for the treatment of pain, inflammation, rheumatism, anxiety and several neurological disorders (Bonini *et al*., 2018). The plant has gained considerable scientific attention owing to its essential oil, which is rich in volatile bioactive constituents possessing diverse pharmacological properties (Pollastro *et al*., 2018). The essential oil is composed predominantly of mono- and sesquiterpenes, with β-caryophyllene, α-humulene, caryophyllene oxide, α-pinene, β-pinene, myrcene, limonene and terpinolene being the major constituents (Novak *et al*., 2001; Zheljazkov *et al*., 2020). These volatile compounds are largely responsible for the characteristic aroma of the plant and contribute significantly to its antioxidant, antimicrobial and anti-inflammatory activities (El-Mernissi *et al*., 2024).

The essential oil of *Cannabis sativa* is characterized by a complex mixture of volatile compounds, accounting for approximately 99.8% of the total oil composition, belonging to various chemical classes, including cannabinoids, terpenoids, flavonoids, alkaloids, steroids, fatty acids and amino acids (Pollastro *et al*., 2018). The oil is composed mainly of monoterpene hydrocarbons (54.0%) and sesquiterpene hydrocarbons (44.2%), with β-caryophyllene, α-humulene, caryophyllene oxide, α-pinene, β-pinene, myrcene, limonene and terpinolene reported as the major constituents. These volatile compounds are responsible for the characteristic aroma of the essential oil and contribute to its biological activities. However, the composition of essential oil varies among cultivars due to differences in genetic makeup and environmental conditions (Novak *et al*., 2001; Zheljazkov *et al*., 2020). The antioxidant potential of *Cannabis sativa* essential oil has been widely attributed to its terpene-rich composition, particularly sesquiterpenes and monoterpenes, which are capable of neutralizing reactive oxygen species and preventing oxidative damage. Experimental studies have demonstrated that the essential oil exhibits dose-dependent free radical scavenging activity together with appreciable ferric ion reducing capacity, indicating its ability to donate hydrogen atoms and electrons to unstable free radicals (Palmieri *et al*., 2021). The essential oil of the plant exert its antibacterial effects primarily by interacting with the phospholipid bilayer of bacterial cell membranes, leading to disruption of membrane integrity, interfere with membrane-bound enzymes involved in respiration and ATP synthesis, thereby impairing bacterial metabolism and inhibiting cell growth.

*Corresponding Author

MATERIALS AND METHODS

Extraction of essential oil: Bhang leaves were collected from the herbal garden of SKUAST-Jammu, Chatha, in the month of July, 2025. Collected leaves were washed thoroughly. Essential oil from fresh leaves was extracted by hydrodistillation using clevenger-type apparatus (Guleria *et al.,* 2008). The leaves were repeatedly subjected to hydrodistillation until no more essential oil was obtained. The obtained oil was collected, dehydrated with anhydrous sodium sulphate and was stored in glass vials at -4°C for further analysis. The weight and amount of oil was determined in order to calculate the percent yield (Fig. 1).The percent yield of essential oil was determined using the following formula:

$$\text{Percent yield} = \frac{\text{Weight of oil collected (in grams)} \times 100}{\text{100 grams of leaf sample}}$$

Determination of antioxidant activity:

The antioxidant activity of the essential oil was evaluated by measuring the ability to scavenge DPPH radicals (Bozin *et al*., 2006). Different concentrations of the essential oil were taken from 10 mg/ml stock solution prepared in methanol and volume was made to 1 ml with methanol. After that 1 ml of the 90 µM DPPH solution (prepared in methanol) and then 2 ml methanol was added to make the final volume of the reaction mixture upto 4ml. The final reaction mixture was shaken well and kept in the dark for 1 hour at room temperature. After incubation, absorbance was taken at 517 nm wavelength by using double beam UV–vis spectrophotometer. Methanol was taken as the negative control whereas the reaction mixture without essential oil was taken as the positive control.

Percentage of radical scavenging activity (% RSA) = $(1\text{-}A_{sample}/A_{blank}) \times 100$

Where, A_{blank} is the absorbance of the control (containing all the reagents except sample) and A_{sample} is the absorbance of the test sample. Absorbance decreases with increase in antioxidant activity. The concentration of the essential oil at which absorbance becomes exactly half of that of the control is known as IC_{50}.Butylated hydroxytoluene (BHT) was used as the reference compound.

The FRAP assay was conducted according to Benzie and Strain (1996). The working FRAP reagent was freshly prepared by mixing 100 ml of sodium acetate buffer (300 mM, pH 3.6), 10ml of 10 mM TPTZ [2,4,6-tri (2-Pyridyl)-S-triazine] solution in 40mM HCl and 10ml of 20 mM $FeCl_3$and 12ml distilled water. To carry out the assay, different concentrations of essential oil was mixed with 3 ml of FRAP reagent. After incubation for 10 min, the absorbance was measured at 593 nm. The FRAP activity was calculated from the calibration curve of ferrous sulphate ($FeSO_4.2H_2O$) and the value of FRAP was expressed as mM $FeSO_4.2H_2O$ equivalents/g of dry weight of the essential oil.

Determination of antibacterial activity:

Bacterial strains used in the present study: The study utilized three-gram positive bacterial strains namely *Bacillus subtilis* MTCC 2389, *Staphylococcus aureus* MTCC 7443 and *Micrococcus luteus* MTCC 4821, as well as two-gram negative strains, *Escherichia coli* MTCC2127 and *Klebsiella pneumoniae* MTCC7172. These strains were procured from IMTECH Chandigarh. The bacterial cultures were preserved in nutrient agar slants and stored at -4^0C. These cultures were used as stock cultures.

Preparation of inoculums: The selected bacterial strains were introduced into a nutrient broth and incubated for 24 hours at a temperature of 37 ^{0}C. The optical density of the resulting active bacterial cultures was adjusted to match the 0.5 Macfarland standard. This was achieved by diluting the cultures with nutrient broth. The turbidity of the cultures was then determined by measuring the absorbance at 600 nm using a spectrophotometer.

Agar-well diffusion method: The antimicrobial activity of the essential oil was evaluated qualitatively using the agar - well diffusion method, as described by Feyza *et al*., 2009, with some modifications. The sterilized nutrient agar (20 ml) was poured into sterile petri plates and allowed to solidify undisturbed in a sterile laminar air flow chamber. Once solidified, the petri plates were uniformly inoculated with 100 µl of a bacterial suspension (10^9 CFU / ml) using a sterile glass spreader. The plates were then left undisturbed for a few minutes to allow the bacterial suspension to be absorbed into the nutrient agar. Wells with a diameter of 6 mm were aseptically created in the agar plates and 6 mg/ml of the essential oil was added to each well. Streptomycin (20 µg/ml) was added to a well as a positive control to test the bacteria's sensitivity and 10 µl of DMSO was added to the wells as a negative control. The plates were then left at room temperature for a few minutes inside the sterile laminar air flow chamber to allow for proper diffusion of the essential oil from their respective wells into the media. Following this, the plates were incubated at 37 ^{0}C for 24 hours. After the incubation period, the zones of inhibition were measured. This method allows for a qualitative assessment of the antimicrobial activity of the essential oil.

Determination of minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) was determined following the method outlined by Wayne, 2003. This involved using the broth dilution method to evaluate the individual MICs of the essential oil against selected bacterial strains. The stock solution of essential oil was prepared by dissolving in DMSO in the 1:1 (v/v) ratio. The final volume was then made to 1 ml with MHB. The essential oil was then diluted serially by using MHB. 100 µl of each dilution of the EOs was transferred to microfuge tubes containing 90 µl of the MHB. 10 µl of bacterial suspension were then added to each dilution of the essential oils, and final volume

was made to 200 µl with MHB. Positive control tubes contained only broth and inoculums, but no essential oil, whereas, only DMSO and inoculum were added in the negative control tubes. The tubes were then incubated at 37°C for 24 hours. After incubation, 40µl of p-iodonitrotetrazolium violet solution was added to examine the bacterial growth for another 20 minutes. The tubes were then examined for colour change. Development of pink colour (due to reduction of dye) indicated the bacterial growth. The lowest concentration of essential oil at which no colour change of the dye took place (or no bacterial growth observed) was considered as the MIC for that particular oil.

Statistical analysis: For each measurement, three replicates of each sample were performed subjected to one way analysis of variance (ANOVA) and significant differences between samples were determined using R studio software (Version, 2018).

RESULTS AND DISCUSSION

The antioxidant activity of *Cannabis sativa* essential oil was evaluated using the DPPH radical scavenging assay. The IC_{50} value of bhang essential oil was found to be 71.99 ± 0.34 µg/ml**,** whereas the standard antioxidant **(**BHT**)** exhibited a significantly lower IC_{50} value of 30.05 ± 0.26 µg/ml, indicating its superior free radical scavenging activity (Table 1). The essential oil demonstrated appreciable antioxidant activity, suggesting the presence of naturally occurring bioactive constituents capable of donating hydrogen atoms or electrons to neutralize DPPH free radicals. These findings indicate that essential oil possesses antioxidant potential and may serve as a natural source of antioxidant compounds. The ferric reducing antioxidant power (FRAP) assay was employed to evaluate the reducing ability of *Cannabis sativa* essential oil. The FRAP value of essential oil was recorded as 374.12 ± 0.31 µM Fe^{2+} equivalents/g of dry weight**,** whereas the standard antioxidant **(**BHT) exhibited a significantly higher FRAP value of 1125.84 ± 0.33 µM Fe^{2+} equivalents/g of dry weight, indicating greater reducing power (Table 1). However, the essential oil exhibited measurable ferric ion reducing ability, demonstrating its antioxidant potential.

The antibacterial activity of bhang essential oil was evaluated against five bacterial pathogens *via* agar well diffusion method. The inhibitory activity of the essential oil against the tested bacterial strains is reflected in Table 2. Among the tested microorganisms, the highest antibacterial activity was observed against *Escherichia coli*, with an inhibition zone diameter of 11.70 ± 0.34 mm, followed by *Micrococcus luteus*, showing an inhibition zone of 11.30 ± 0.11 mm. The essential oil also inhibited the growth of *Klebsiella pneumoniae, Staphylococcus aureus* and *Bacillus subtilis*, producing inhibition zone diameters of 10.30 ± 0.26 mm, 10.00 ± 0.23 mm and 8.70 ± 0.26 mm, respectively (Plate 1). Streptomycin (2 µg), used as the positive control, exhibited significantly larger inhibition zones against all bacterial strains, whereas DMSO (99.9%), used as the negative control. These findings indicate that the essential oil possesses moderate antibacterial activity against both gram-positive and gram-negative bacteria, although its efficacy varied among the tested bacterial species. The minimum inhibitory concentration (MIC) of essential oil against the susceptible bacterial strains ranged from 1500 to 5000 µg/ml (Table 3). The lowest MIC value (1500 µg/ml) was recorded against *Escherichia coli*, indicating that this organism was the most susceptible to the essential oil. This was followed by *Micrococcus luteus*, which exhibited an MIC of 2000 µg/ml, while *Klebsiella pneumoniae* showed an intermediate MIC value of 3500 µg/ml. Higher MIC values of 4500 µg/ml and 5000 µg/ml were observed against *Staphylococcus aureus* and *Bacillus subtilis*, respectively, indicating comparatively lower sensitivity. Overall, the MIC values were in agreement with the agar well diffusion assay, where larger inhibition zones corresponded to lower MIC values, confirming that the essential oil possesses moderate antibacterial activity against both gram-positive and gram-negative bacteria.

The data was compared with literature values and it was found that the data was in good agreement with the published data. At a concentration of 500 µg/ml, the essential oil has been reported to exhibit 76.54% DPPH radical scavenging activity and 56.10% FRAP activity, indicating antioxidant capacity. In comparison, the lower DPPH IC_{50} value obtained in the present study reflects a relatively higher free radical scavenging efficiency (Shami *et al*., 2023). The observed difference between the DPPH and FRAP assays suggests that the bioactive compounds present in *Cannabis sativa* essential oil, particularly cannabinoids and terpenes, possess a stronger ability to neutralize stable free radicals via a single-electron transfer mechanism than to reduce ferric (Fe^{3+}) ions into ferrous (Fe^{2+}) ions (Hacke *et al*., 2019). The antioxidant capacity varied among cultivars due to differences in the concentration of major volatile constituents, particularly β-caryophyllene, α-humulene and myrcene (Palmieri *et al*., 2021). Essential oils obtained from different *Cannabis sativa* cultivars have been reported to exhibit antibacterial activity against multi drug-resistant *Staphylococcus pseudintermedius*. The antimicrobial effect was associated with the synergistic interaction between cannabinoids and terpenes, with cultivars containing higher levels of phytocannabinoids demonstrating greater antibacterial efficacy (MIC 23.81 mg/mL; MBC 47.61 mg/mL) than those with lower phytocannabinoid content (MIC 47.61 mg/mL; MBC 95.22 mg/mL) (Pieracci *et al*., 2024). The antibacterial activity is due to hydrophobic constituents of the essential oil, which disrupt

bacterial cell membranes, increase membrane permeability, promote leakage of intracellular contents, and ultimately inhibit microbial growth (Nafis *et al*., 2019).

Table 1. Antioxidant activity of *Cannabis sativa*

Test material	**DPPH Radical Scavenging IC_{50} (µg/ml)**	**FRAP (µM Fe^{2+} equivalents/g of dry wt.)**
Essential oil	71.99 ± 0.34µg/ml (IC_{50})	374.12 ± 0.31
BHT	30.05 ± 0.26	1125.84 ± 0.33

Data is Mean ± S.D. of three replications

Table 2. Antibacterial activity *via* agar well diffusion method of *Cannabis sativa*

Test material	**Conc.**	**Gram positive bacteria**			**Gram negative bacteria**	
		Bacillus subtilis	***Staphylococcus aureus***	***Micrococcus luteus***	***Escherichia coli***	***Klebsiella pneumoniae***
Essential oil	6 mg/ml	8.60 ± 0.24 mm	9.80 ± 0.20 mm	10.20 ± 0.25 mm	**11.50 ± 0.28 mm**	8.90 ± 0.24 mm
Streptomycin (+ve) control	2 µg/ml	20.00± 0.21 mm	20.00± 0.24 mm	18.00± 0.23 mm	18.00± 0.25 mm	17.00 ± 0.26
DMSO-99.9% (-ve) control	10 µl	ND	ND	ND	ND	ND

Data is Mean ± S.D. of three replications
ND: Activity not determined at tested concentration

Table 3. Minimum Inhibitory Concentration (MIC) of *Cannabis sativa*

Essential oil	**MIC (µg/ml)**				
	Bacillus subtilis	***Staphylococcus aureus***	***Micrococcus luteus***	***Escherichia coli***	***Klebsiella pneumoniae***
Essential oil	5000	4500	2000	1500	3500
Streptomycin (+ve) control	2	2	2	2	2

Data is Mean of three replications
ND: Activity not determined at tested concentration

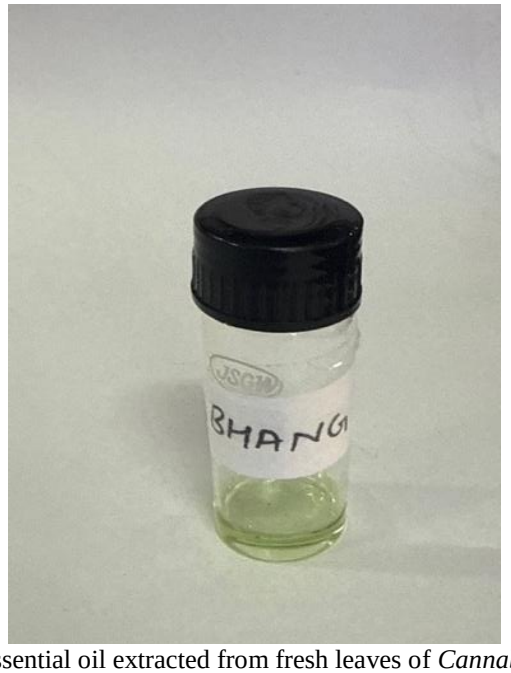

Fig. 1. Essential oil extracted from fresh leaves of *Cannabis sativa*

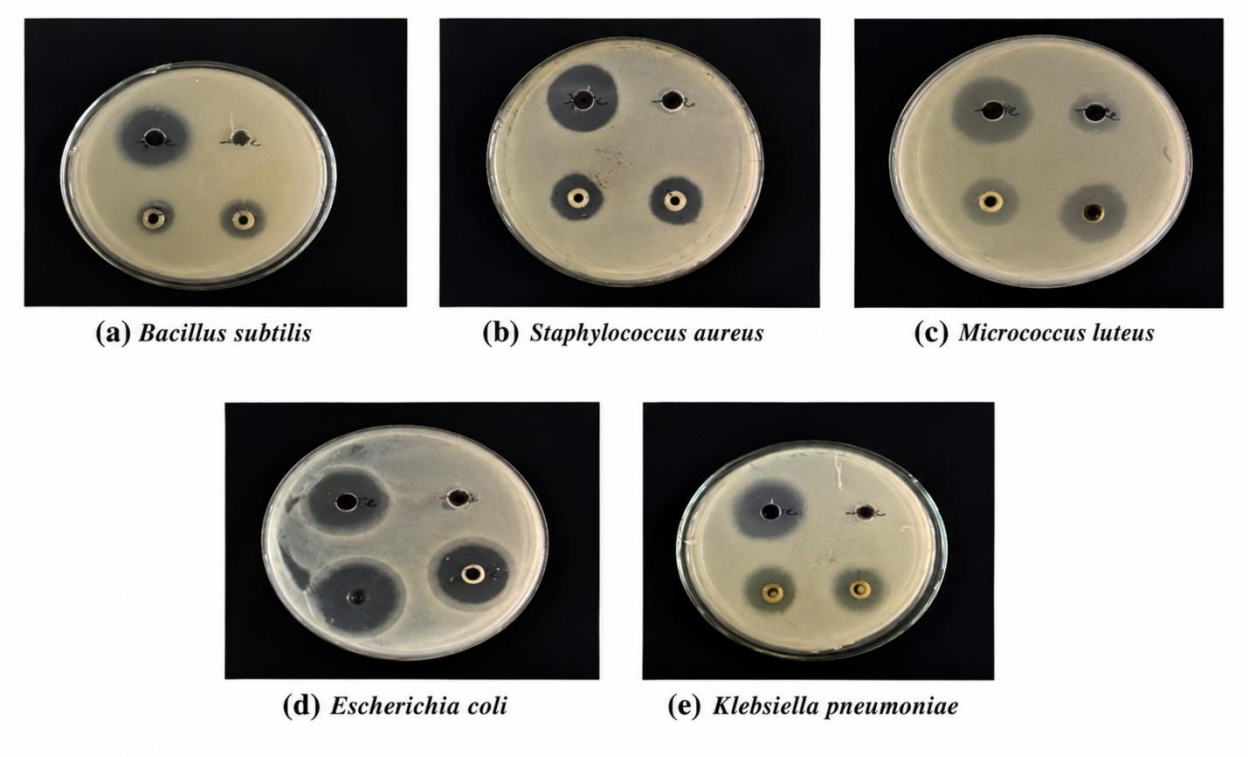

Plate 1. Antibacterial activity of *Cannabis sativa* against a) *Bacillus subtilis* MTCC2389 b) *Staphylococcus aureus* MTCC7443 c) *Micrococcus luteus* MTCC4821 d) *Escherichia coli* MTCC2127 e) *Klebsiella pneumonia* MTCC7172 bacterial strains.

The findings of the present study demonstrated that *Cannabis sativa* essential oil possesses promising antioxidant and broad-spectrum antibacterial activities, highlighting its potential as a valuable natural source of bioactive compounds. The essential oil effectively scavenged free radicals and inhibited the growth of both gram-positive and gram-negative bacterial strains, which may be attributed to the combined action of cannabinoids, terpenes and other phenolic constituents. These compounds are known to neutralize reactive oxygen species, reduce oxidative stress and disrupt bacterial cell membranes, thereby inhibiting microbial growth and proliferation. Owing to these multifunctional biological properties, bhang essential oil shows considerable potential for application as a natural antioxidant and antimicrobial agent in pharmaceutical formulations, food preservation systems and other health-related products as a safer alternative to synthetic additives.

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RESEARCH ARTICLE

FLORISTIC DIVERSITY AND SPECIES DISTRIBUTION IN DIFFERENT FOREST STANDS IN NORTHERN CHHATTISGARH, INDIA

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Abstract: The understory or vegetation beneath the forest canopy, or undergrowth, flourishes under the stratum of the forest stand and has significant distribution and impact on diversity, regeneration, nutrient cycling, and ecological functions. These are diverse life forms and have a substantial role in forest ecology, silvicultural aspects, and wildlife perspectives. Generally, the occurrence, distribution, and diversity of understory depend upon the canopy closure, light availability, site quality, and environmental conditions that can favor or limit it in a particular region. The present investigation was carried out in five forest land uses (i.e., degraded forest, moderately dense forest, regenerated forest, dense forest, and teak plantation) in the Duldula region of northern Chhattisgarh. Stratified simple random sampling techniques were used for data collection. A total of 89 species (28 tree species, 14 shrub species, 17 climber species, and 30 herb species) were recorded in different forest stands of the Duldula region of Jashpur (C.G.). In the overall floristic richness tree, shrub, climber, and herb contribute 31.46%, 15.73%, 19.10%, and 33.71%, respectively, in total. The frequency class distribution showed mostly species restricted to Classes A and B, reflecting their infrequent or moderate distribution. The origin of species or nativity analysis reflects that most of the species that occurred in different forest stands are native to India and South Asia. These floristic resources need to be preserved, conserved, and protected for future generations and to achieve sustainability.

Keywords: Distribution, Floristic, Flora, Diversity, Regeneration

INTRODUCTION

Forests are the most diverse and biologically rich ecosystems that support human wellbeing and their existence. Floristic representation of forest ecosystems is the key indicator of resilience and health. The forest composition reflects the assemblage of plant species in a given environmental setting, with specific associations among the diverse flora (Poudel and Devkota, 2022; Devi *et al.,* 2023; Tomar *et al.,* 2008 and Tomar, 2024). Species diversity is the variation of the stand represented by its assemblage among the plant community that affects and maintains the forest ecosystem structure and function (Tilman and Dowing, 1994; Jhariya *et al.,* 2024). Forest health is regulated by natural recovery through regeneration in space and time, which determines the status of future stands and their overall sustainability (Jhariya *et al.,* 2023).

Understanding the distribution, pattern, and species presence in different stands is key to assessing the vegetation dynamics, environmental gradient, and disturbance regimes. This information helps to develop management and conservation strategies and plan towards sustainability (Baghel *et al.,* 2025; Rajput *et al.,* 2025). The northern part of Chhattisgarh in India shows the tropical deciduous forest landscape with substantial diversity and richness of biological resources. Despite their socio-economic-ecological role, these resources are degrading day by day due to biotic interferences and mismanagement. However, various scientific explorations have documented the species composition of central Indian forests, but a comprehensive database of northern Chhattisgarh is still a scarce resource and remains limited. Thus, this study highlights the floristic diversity, richness, distribution, and composition in different forest stands in line to facilitate a baseline database towards biodiversity conservation, restoration, and sustainable resource management.

MATERIALS AND METHODS

The study sites placed at Duldula block of Jashpur district. The study was conducted in the Duldula region of Northern Chhattisgarh. The study area lies between 22˚17-23˚15 N and 83˚30-84˚24 E. The forest of the area is tropical dry deciduous type with domination of *Shorea robusta*. The climate is monsoon type with 3-4 months of critical dry periods. Several economically and medicinally important plants occur in the forest area. Highly mountainous and forested, Jashpur is known for its natural beauty and is rich in natural resources; the land is fertile, and forest products are in abundance.

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The forests of the Duldula region of northern Chhattisgarh are divided into different sites based on disturbance and canopy closure, *viz*., degraded forest, regenerated forest, moderately dense forest, dense forest, and teak plantation. Following local inquiry, the field investigation was done, followed by site condition monitoring. The vegetation (trees, saplings, seedlings, shrubs, climbers, and herbs) was identified through local inquiry with people, consulting the literature of the region. The data were collected, recorded, and quantified at the species level. The trees and saplings were analysed by randomly laying quadrats of size 10 m x 10 m, while the seedlings, shrubs, and climbers were assessed within the 5 m x 5 m quadrats in each site. The herb was assessed using a 50 cm x 50 cm quadrat. The seedlings and shrubs were measured at the basal region, while the climbers and herb were enumerated by the count method. The assessment of species rarity or commonness in the sanctuary area was conducted using frequency classes. In this classification, the percentage frequency of the species was classified as A, B, C, D, and E, where A represents rare, B represents low frequency, C represents intermediate frequency, D represents moderately high frequency, and E represents high frequency (common) (Raunkiaer, 1934). Further, the nativity of the species and utilization were recorded using standard literature.

RESULTS AND DISCUSSION

Floristic richness and diversity

A total of 21 tree species in different forest stands were recorded. A total of 5, 3, 7, 15, and 3 species under the tree layer were recorded for degraded forest stand, regenerated forest, moderately dense forest, dense forest, and teak plantation, respectively (Table 1). In the case of the sapling layer, a total of 7, 15, 13, 16, and 3 species were found in the degraded forest stand, regenerated forest, moderately dense forest, dense forest, and teak plantation, respectively. A total of 8, 10, 14, 16, and 5 species were recorded in the degraded forest stand, regenerated forest, moderately dense forest, dense forest, and teak plantation for the seedling layer. A total of 14 shrub species were found in different forest stands, and the highest number of species was reported in regenerated forest. A total of 17 climber species were recorded in different forest stands, and the highest number of species was recorded both for regenerated forest and dense forest. In the herb layer, a total of 30 species were recorded, and the highest number of species (14) was recorded for dense forest.

Table 1: Presence/Occurrence of the tree species in different sites

Species	Degraded Forest	Regenerated Forest	Moderately Dense Forest	Dense Forest	Teak Plantation
Tree Layer					
Anogeissus latifolia Wall.	-	-	+	-	-
Adina cardifolia Hook.f.	-	-	-	+	-
Boswellia serrata, Roxb.	-	-	+	-	-
Buchanania lanzan Spreng.	+	+	+	+	-
Cassia fistula Linn.	-	-	-	-	+
Chloroxylon Swietenia D.C.	-	-	-	+	-
Diospyros melanoxylon Roxb.	+	-	+	+	-
Gardenia latifolia Roxb.	-	-	+	+	-
Lagerstroemia parviflora Roxb.	-	-	+	-	+
Litsea glutinosa (Lour.) C.B.Rob	-	+	-	+	-
Madhuca latifolia, Roxb.	+	-	-	+	-
Phyllanthus emblica L.	-	-	-	+	-
Pterocarpus marsupium, Roxb.	-	-	-	+	-
Saccopetalum tomentosum H.F, & Thoms	-	-	-	+	-
Schleichera oleosa Willd.	-	-	-	+	-
Shorea robusta Gaertn.f.	+	-	+	+	-
Syzygium cumini (L.)	-	-	-	+	-
Tectona grandis Linn. f.	-	-	-	-	+
Terminalia bellerica Roxb	-	-	-	+	-
Terminalia chebula Retz.	-	-	-	+	-
Terminalia tomentosa W&A.	+	+	-	-	-
Sapling Layer					
Anogeissus latifolia Wall.	-	-	+	+	-
Antidesma ghaesembilla, Gaertn.	-	+	-	-	-
Buchanania lanzan Spreng.	+	+	+	+	-

Cassia fistula Linn.	-	-	-	+	+
Cleistanthus collinus, Benth.	-	-	+	-	-
Diospyros melanoxylon Roxb.	+	+	+	+	+
Gardenia latifolia Roxb.	-	-	+	-	-
Grewia tillifolia,Vahl.	-	+	-	-	-
Holarrhena pubescens Wall.	+	+	+	+	-
Kydia calycina Roxb.	-	-	+	-	-
Lagerstroemia speciosa (L.) Pers.	-	-	-	+	-
Madhuca latifolia Roxb.	+	+	+	+	-
Mitragyna parviflora Roxb.	-	+	-	+	-
Phyllanthus emblica L.	-	-	-	+	-
Pterocarpus marsupium Roxb.	-	+	-	+	-
Schrebera swietenioides Roxb.	-	+	-	-	-
Semecarpus anacardium, L.f.	-	-	+	-	-
Shorea robusta Gaertn. f.	+	+	+	+	-
Stereospermum chelenoides (L. fil.) DC.	-	+	-	-	-
Strychnos potatorum Linn.	+	+	-	+	-
Syzygium cumini (L.)	-	+	+	+	-
Tectona grandis Linn. f.	-	-	-	-	+
Terminalia bellerica Roxb	-	-	-	+	-
Terminalia chebula Retz.	-	+	+	+	-
Terminalia tomentosa W&A.	+	+	+	+	-
Seedling Layer					
Adina cardifolia Hook.f.	-	-	-	+	-
Anogeissus latifolia Wall.	-	-	+	+	-
Antidesma ghaesembilla Gaertn.	-	+	-	-	-
Buchanania lanzan Spreng.	+	+	+	+	-
Careya arborea Roxb.	+	-	+	-	-
Cassia fistula Linn.	-	-	-	+	+
Diospyros melanoxylon Roxb.	+	+	+	+	+
Grewia tillifolia Vahl.	-	+	+	-	-
Holarrhena pubescens Wall.	+	+	+	+	+
Kydia calycina Roxb.	-	-	-	-	-
Lagerstroemia parviflora Roxb.	-	-	+	+	-
Madhuca latifolia Roxb.	-	+	+	+	-
Ougeinia oojeinensis Benth.	-	-	-	+	-
Phyllanthus emblica L.	-	+	+	+	-
Pterocarpus marsupium Roxb.	-	-	+	+	-
Schleichera oleosa Willd.	-	-	-	+	-
Semecarpus anacardium, L.f.	-	+	+	-	-
Shorea robusta Gaertn.f.	+	+	+	+	+
Strychnos potatorum Linn.	+	-	-	-	-
Syzygium cumini (L.)	+	-	+	+	-
Tectona grandis Linn. f.	-	-	-	-	+
Terminalia chebula Retz.	-	-	-	+	-
Terminalia tomentosa W&A.	+	+	+	+	-
Shrub Layer					
Antidesma diandrum Roth.	+	-	-	-	-
Calotropis gigantea R. Br.	-	-	-	-	+
Carissa spinarum A. DC.	-	+	+	+	-
Casearia graveolens Dalz.	+	+	-	+	-
Caesalpinia crista Linn.	-	+	-	-	-
Capparis aphylla Roth.	-	-	+	-	-
Embelia robusta Roxb	-	-	-	+	-
Indigofera pulchella Roxb.	+	-	+	-	-
Indigofera tinctoria Linn.	-	+	-	-	-
Lantana camara Linn.	+	+	-	-	+

Leea asiatica (L.) Ridsdale	-	+	-	-	-
Woodfordia floribunda Salist.	+	-	+	+	-
Ziziphus oenoplia Mill.	+	+	-	-	-
Ziziphus xylopyra Willd.	-	+	+	+	+
Climber Layer					
Acacia caesia W. & A.	+	-	+	+	-
Abrus precatorius Linn.	+	+	+	+	-
Acacia pennata Willd.	+	-	-	-	-
Atylosia scarabaeoides (L.) Benth	-	+	+	-	-
Bauhinia valhii W. & A.	+	-	+	+	-
Butea superba Roxb.	+	-	-	-	+
Cajanus scarabaeoides (L.) Thouars	-	-	-	-	+
Calycopteris floribunda Lamk.	-	+	-	-	-
Cayratia auriculata (Roxb.) Gamble	-	-	-	-	-
Combretum decandrum, Roxb.	-	+	-	-	-
Cryptolepis buchanani R. & S.	-	+	-	-	-
Cuscuta reflexa Roxb.	+	-	-	-	-
Derris scandens Benth.	-	-	-	-	+
Hemidesmus indicus R. Br.	-	-	-	+	-
Smilax ovalifolia Rox	-	-	-	+	-
Olax imbricata Roxb	-	-	-	-	+
Ventilago calyculata Tul.	-	+	+	+	-
Herb Layer					
Ageratum conyzoides (L.)	-	+	-	-	-
Andrographis paniculata Burm.f.	-	-	-	+	-
Apluda mutica L.	-	-	-	+	-
Apluda varia Hack	+	-	+	+	-
Blepharis maderaspatensis (L.) B.Heyne ex Roth	-	+	-	-	-
Cassia absus L.	-	-	-	+	-
Cassia tora L.	-	-	+	+	-
Chrolophytum tuberosum Roxb.	-	-	-	+	-
Chrysopgon montanus Trin.	-	-	-	+	-
Crotalaria retusa Linn.	-	+	-	-	-
Cynodon dactylon Pers.	+	-	+	+	+
Cyprus rotundus L.	+	+	+	+	+
Desmostachya bipinnata Stapf.	-	-	+	-	-
Echinochloa colona Link.	-	-	+	-	-
Evolvulus nummularius (L.)	-	-	-	-	+
Eragrostis diarrhena (Schult. & Schult.f.) Steud.	+	+	+	+	-
Heteropogon contortus Beauv.	+	-	-	-	-
Imperata cylindrica P. Beauv.	+	-	-	-	-
Leucas aspera (Willd.) Link	-	-	-	-	+
Malvastrum coromandelianum L.	-	-	-	-	+
Oplismenus burmannii (Retz.) P.Beauv	-	+	+	+	-
Phyllanthus fraternus Webster	-	-	-	-	+
Psoralea corylifolia Linn.	-	+	-	-	+
Pteris vittata L.	+		+	-	+
Rungia parviflora Nees	-	+	-	-	-
Saccharum spontaneum L.	-	-	-	+	-
Setaria glauca L.	-	-	+	+	-
Themeda quadrivalvis O. Kuntz.	-	+	+	-	-
Trifolium sp	+	+	-	+	+
Urtica dioica Linn.	-	+	-	-	-

Note: (+) = present, (-) = absent

A total of 89 species (28 tree species, 14 shrub species, 17 climber species, and 30 herb species) were recorded in different forest stands of the Duldula region of Jashpur (C.G.). In the overall floristic richness tree, shrub, climber, and herb contribute 31.46%, 15.73%, 19.10%, and 33.71%, respectively, in total. The highest contribution by herbaceous vegetation reflects the suitable microclimate, followed by canopy gaps that permit light to reach the ground and provide a suitable growing environment for the species. Herb species are highly responsive to disturbance and environmental regimes, making them a reliable indicator of ecosystem condition and forest recovery (Gilliam, 2007). After herbaceous vegetation, tree species possess higher flora, reflecting their dominant position in forest structure, maintaining the upper canopy and ecological services (Pretzsch, 2009). Climbers contribute to structural complexity and species interactions in tropical forest stands. They enhance vertical connectivity and biodiversity of the region (Schnitzer and Bongers, 2002). The lower comparative representation of shrubs than others might be a result of competition, canopy architecture, and site conditions (Odum and Barrett, 2005).

Species Rarity and Commonness

The frequency class distribution entails the species rarity and commonness over the site (Table 2). The Raunkiaer class varied among different forest stands and vegetation layers, which showcases the variation in species admixture and regeneration prospects. In the degraded forest, tree layers reflect the absence of intermediate-sized classes; 40% of species showed rarity over the site, while low, moderately high, and common classes showed equal distribution (each 20%). In contrast, regenerated, moderately dense, and dense forests reflect higher concentrations of saplings, seedlings, and herb distribution in Class B, showing more stable vegetation. Under teak plantation, it reflects simpler distribution from lower to higher classes. The most common frequency class distribution is restricted to Classes A and B, reflecting its infrequent or moderate distribution, which is a common characteristic of tropical forests (Condit *et al.*, 2002).

Table 2: Frequency class distribution of species under different strata and sites

Site	Layer	Frequency class				
		A	**B**	**C**	**D**	**E**
Degraded Forest	Tree	2	1	0	1	1
	Sapling	4	1	1	1	0
	Seedling	3	2	0	2	1
	Shrub	0	3	3	0	0
	Climber	0	2	3	0	0
	Herb	0	4	3	1	0
Regenerated Forest	Tree	2	1	0	0	1
	Sapling	6	6	1	1	1
	Seedling	3	4	2	1	0
	Shrub	1	6	1	0	0
	Climber	2	2	1	0	1
	Herb	0	3	6	2	0
Moderately Dense Forest	Tree	2	6	1	0	1
	Sapling	3	3	4	2	1
	Seedling	2	6	4	1	1
	Shrub	0	3	2	0	0
	Climber	1	1	1	1	1
	Herb	1	8	2	1	0
Dense Forest	Tree	10	4	1	0	1
	Sapling	5	6	3	1	1
	Seedling	1	10	0	4	1

	Shrub	1	3	1	0	0
	Climber	0	4	1	0	1
	Herb	1	9	4	0	1
Teak Plantation	Tree	2	0	0	0	1
	Sapling	2	0	0	0	1
	Seedling	1	1	1	2	0
	Shrub	1	1	0	0	1
	Climber	3	2	0	0	0
	Herb	3	3	1	1	0

Note: A (0-20)= rare, B (20-40)= low, C (40-60)= intermediate, D (60-80)= moderately high, E (80-100)= high/common

Nativity analysis

The origin of species or nativity analysis reflects that most of the species that occurred in different forest stands are native to India and South Asia, reflecting strong indigenous characters of the floristic diversity (Table 3). Further, a few exotic taxa were found in different layers, showcasing natural floristic integrity retained by the study forests besides a limited introduced species distribution. The dominance of indigenous taxa in northern Chhattisgarh imitates natural phytogeographical affinity, ecosystem stability and resilience (Pavoine and Ricotta, 2023).

Table 3: Nativity of species found in the study sites of northern Chhattisgarh

Tree Species		
Species	**Family**	**Origin**
Adina cordifolia Hook.f.	Rubiaceae	Southern Asia
Anogeissus latifolia Wall.	Combretaceae	India
Antidesma ghaesembilla Gaertn.	Euphorbiaceae	Australia
Boswellia serrata Roxb.	Burseraceae	India
Buchanania lanzan Spreng.	Anacardiaceae	South east Asia
Careya arborea Roxb.	Lecythidaceae	Indochina
Cassia fistula Linn.	Legumuinosae	India
Cleistanthus collinus Benth.	Phyllanthaceae	Australia
Chloroxylon Swietenia D.C.	Meliacese	Southern India
Diospyros melanoxylon Roxb.	Ebenaceae	India
Gardenia latifolia Ait.	Rubiaceae	India
Grewia tillifolia Vahl.	Tiliaceae	Sri Lanka
Holarrhena pubescens Wall.	Apocynaceae	Southern Africa
Kydia calycina Roxb.	Juncaceae	South east Asia
Lagerstroemia speciosa (L.) Pers.	Lytharaceae	Southern Asia tropical
Litsea glutinosa (Lour.) C.B.Rob	Lauranceae	India
Madhuca latifolia Roxb.	Sapotaceae	India
Mitragyna parviflora Roxb.	Rubiaceae	India
Ougeinia oojeinensis Benth.	Leguminosae	India
Phyllanthus emblica L.	Euphorbiaceae	India
Pterocarpus marsupium Roxb.	Legumuinosae	West indies
Saccopetalum tomentosum H.F,&Thoms	Anonaceae	India, Sri Lanka
Schleichera oleosa Willd.	Sapindaceae	Indo China
Schrebera swietenioides Roxb.	Dipterocarpaceae	India
Semecarpus anacardium L.f.	Anacardiaceae	India
Shorea robusta Gaertn. f.	Dipterocarpaceae	India
Stereospermum chelenoides (L. fil.) DC.	Bignoniaceae	South Asia
Strychnos potatorum, Linn.	Loganiaceae	Burma
Syzygium cumini (L.)	Sapindaceae	India
Tectona grandis Linn. f.	Lamiaceae	South East Asia
Terminalia bellerica Roxb	Combretaceae	South East Asia
Terminalia chebula Retz.	Combretaceae	South Asia

Terminalia tomentosa W&A.	Combretaceae	Southern & Southeast Asia
Shrub Species		
Antidesma diandrum, Roth.	Phyllanthuceae	China, Pakistan
Calotropis gigantea R. Br.	Apocynaceae	Shri Lanka, India
Carissa spinarum A. DC.	Apocynaceae	Africa
Casearia graveolens Dalz.	Salicaceae	Asia, China
Caesalpinia crista Linn.	Fabaceae	India, Africa
Capparis aphylla Roth.	Capparaceae	India, Africa
Embelia robusta Roxb	Leguminoseae	India
Indigofera pulchella, Roxb.	Fabaceae	India
Indigofera tinctoria Linn.	Fabaceae	India
Lantana camara Linn.	Verbenaceae	American tropics
Leea asiatica (L.) Ridsdale	Vitaceae	West Asia
Woodfordia floribunda Salist.	Lythraceae	Shri Lanka, India
Ziziphus oenoplia Mill.	Rhamnaceae	Bangladesh
Ziziphus xylopyra Willd.	Rhamnaceae	Southeast Asia
Climber Species		
Acacia caesia W. & A.	Leguminosae	Bangladesh
Abrus precatorius Linn.	Fabaceae	India
Acacia pennata Willd.	Fabaceae	South-east Asia
Atylosia scarabaeoides (L.) Benth	Fabaceae	Australia
Bauhinia valhii W. & A.	Fabaceae	India
Butea superba Roxb.	Fabaceae	India
Cajanus scarabaeoides (L.) Thouars	Fabaceae	Africa
Calycopteris floribunda Lamk.	Combertaceae	Bangladesh, India
Cayratia auriculata (Roxb.) Gamble	Vitaceae	Africa, Asia
Combretum decandrum Roxb.	Combertaceae	India
Cryptolepis buchanani R. & S.	Apocynaceae	India
Cuscuta reflexa Roxb.	Convolvulaceae	India
Derris scandens Benth.	Fabaceae	India, Asia, Australia
Hemidesmus indicus R. Br.	Apocynaceae	India
Smilax ovalifolia Rox	Smilacaceae	China tropical
Olax imbricata Roxb	Olacaceae	India
Ventilago calyculata Tul.	Rhamnaceae	South-east Asia, Australia
Herb Species		
Ageratum conyzoides (L.)	Asteraceae	Tropical America
Andrographis paniculata Burm.f.	Acanthaceae	India, Shri Lanka
Apluda mutica L.	Poaceae	Central Asia, China, Japan
Apluda varia Hack	Poaceae	Asia, India
Blepharis maderaspatensis (L.) B.Heyne ex Roth	Acanthaceae	Africa, Southeast Asia
Cassia absus L.	Fabaceae	India, Southeast Asia
Cassia tora L.	Fabaceae	Central America
Chrolophytum tuberosum Roxb.	Asparagaceae	Africa, India
Chrysopogon montanus Trin.	Poaceae	India, Southeast Asia
Crotalaria retusa Linn.	Asparagaceae	Tropical Asia, Africa, Australia
Cynodon dactylon Pers.	Poaceae	Africa
Cyprus rotundus L.	Cyperaceae	Africa, Central Europe, Southern Asia
Desmostachya bipinnata, Stapf.	Poaceae	Northern Africa, China, India
Echinochloa colona, Link.	Poaceae	Tropical Asia
Evolvulus nummularius (L.)	Convolvulaceae	Mexico, South America
Eragrostis diarrhena (Schult. & Schult.f.) Steud.	Poaceae	Tropical Africa, Subtropical Asia
Heteropogon contortus Beauv.	Poaceae	Southern Asia, Africa, Northern Australia
Imperata cylindrica P. Beauv.	Poaceae	Southern Asia, India, Australia
Leucas aspera (Willd.) Link	Lamiaceae	India
Malvastrum coromandelianum L.	Malvaceae	North and South America
Oplismenus burmannii (Retz.) P.Beauv	Poaceae	Zimbabwe

Phyllanthus fraternus Webster	Phyllanthus	Pakistan
Psoralea corylifolia Linn.	Fabaceae	India, Shri Lanka
Pteris vittata L.	Pteridaceae	Asia, Tropical Africa, Australia
Rungia parviflora Nees	Acanthaceae	India
Saccharum spontaneum L.	Poaceae	India
Setaria glauca L.	Poaceae	India
Themeda quadrivalivis O. Kuntz.	Poaceae	India, Nepal, Malaysia
Trifolium sp.	Fabaceae	Central Asia
Urtica dioica Linn.	Urticaceae	Europe, Asia, North Africa

Ethnobotanical Scenarios

The ethnobotanical inventory reflects that the recorded tree species are associated with diverse taxonomic groups, with varied parts used for economic and medicinal purposes. The plant part used reflects that bark was the most used than the other parts, showing a strong reliance on this for traditional healthcare. The medicinal utilization is predominantly associated with the cure of gastrointestinal disorders and peptic ulcers using the species *A. cordifolia*, *A. latifolia*, *H. pubescens*, *T. bellirica*, *T. tomentosa*, *S. cumini*, and *P. emblica* (Table 4). Besides, several other medicinal uses (skin care, wound healing, pain, diabetes, various disorders, etc.) were also mentioned with the suitable species. The higher use of bark and leaves in traditional cure reflects their higher preference due to the availability of secondary metabolites in these parts (Yadav *et al*., 2015; Painkra *et al.*, 2015; Tomar and Singh, 2006; Tomar, 2007; Tomar, 2009; Tomar, 2022 & Tomar, 2023).

Table 4: Species utilization and ethnobotanical uses of species

Tree Species	**Plant Part Use**	**Utilization**
Adina cardifolia Hook.f.	Bark	Bark for dysentery, bruises and wounds
Anogeissus latifolia Wall.	Bark, Heart Wood, Gum	Diarrhea, dysentery, skin diseases, jaundice
Antidesma ghaesembilla Gaertn.	Leaf, Branch, Fruit	Fevers, pain, gastrointestinal, timber and dye
Boswellia serrata Roxb.	Gum	Relieves joint pain, asthma & respiratory care, perfumery & cosmetics, paints, varnishes & inks
Buchanania lanzan Spreng.	Root, Leaves	Skin diseases, heal open wounds, cosmetics & soaps, food
Cassia fistula Linn.	Bark, Fruit pulp and Pods, Root, Leaves	Natural laxative, anti-inflammatory agent, constipation, joint pain, and timber source
Chloroxylon swietenia D.C.	Leaf, Stem, Bark	Skin infection treatments, insect-repellent essential oils, fungal skin infections, rheumatism, and painful joints
Diospyros melanoxylon Roxb.	Leaves, Bark, Root, Fruit	Digestive issues, skin eruptions, joint pain, and snakebites. Provides valuable leaves for the rural beedi industry, edible fruits
Gardenia latifolia Roxb.	Bark, Fruit, Timber	Anti-inflammatory, wound-healing, and natural coloring properties, natural dye, perfume, wood crafting
Grewia tillifolia Vahl.	Stem, Bark, Leaves	Healing wounds and treating ulcers
Holarrhena pubescens Wall.	Seed, Bark, Root	Treating severe diarrhea, amoebic dysentery, and skin conditions
Kydia calycina Roxb.	Leaf, Bark, Stem	Treating body pains, skin diseases, and joint aches
Lagerstroemia parviflora Roxb.	Bark, Wood, Leaf	Treat skin sores, carbuncles, and syphilis, coughs, asthma, and bronchitis**,** silk farming, dyes and tannins, fuel and gum
Litsea glutinosa (Lour.) C.B.Rob	Seed, Bark, Leaf, Flowers	Bone fractures and bruises, gastrointestinal relief, incense, agarbatti binding, soap and candle production
Madhuca latifolia Roxb.	Seed Oil, Leaves, Flowers, Bark	Soaps, traditional liquors and industrial alcohol, joint pain, rheumatism, and swelling

Mitragyna parviflora Roxb.	Leaf, Bark	Pain relief**,** wound healing**,** and timber production
Phyllanthus emblica L.	Fruit, Bark	Treats hyperacidity, peptic ulcers, and helps regulate blood sugar levels in diabetic care; immune support, high vitamin C and tannin content
Pterocarpus marsupium Roxb.	Bark, Leaves, Gum, Wood	Timber, dyes, tannins, cosmetics, diarrhea, dysentery, and oral ulcers
Saccopetalum tomentosum H.F,&Thoms	Seed, bark, leaves	Essential oils, carbohydrates, proteins, and essential vitamins
Schleichera oleosa Willd.	Seed, Bark, Leaves	Treat acne, skin itches, burns, and ringworm, relieve rheumatic and joint pains, biodiesel and fuel, lac production
Shorea robusta Gaertn.f.	Seed, Bark, Leaves, Resin	Diarrhea, dysentery, and weak digestion, oil and resin, cooking, making soaps
Stereospermum chelenoides (L. fil.) DC.	Root, Bark, Leaves	Anti-inflammatory, antimicrobial, and timber properties
Strychnos potatorum Linn.	Seed, Fruit	Eye to treat conjunctivitis, corneal ulcers, and watering eyes, paper and textiles
Syzygium cumini (L.)	Seed, Bark, Leaves, Fruit, Wood	Helps lower blood glucose, diarrhea, dysentery, and stomach cramps
Tectona grandis Linn. f.	Bark, Leaves, Fruit	Treating wounds, skin diseases, easing inflammation, blood purifier
Terminalia bellerica Roxb	Fruit /Pulp	For digestive trouble
Terminalia chebula Retz.	Fruit	Seeds for leucorrhea
Terminalia tomentosa W&A.	Bark, Wood	Diarrhea, dysentery, ulcers, fractures, and bleeding disorders, timber, sericulture, tannin and gum

CONCLUSION

The studied forest has high species presence and distribution reflecting a good regional biodiversity. Overall, the floristic diversity recorded in the Duldula region reflects its ecological significance and its utilization in traditional health care, economic and other uses. Considering the dominance of indigenous species and the limited distribution of many taxa, effective protection, conservation, and sustainable management are essential to preserve this valuable floristic diversity for future generations and to ensure long-term ecological resilience and sustainability.

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