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The full manuscript should contain first page with full author names, affiliation, ORCID No and the corresponding author email / ORCID details, followed by full text of the MS file in word format. All data/tables/figures/Images (images must be submitted with the MS in high print-reproducible resolution).

Introduction: This section must provide a brief review of literature, purpose of the study, objectives and the rationale of the research undertaken should be given with proper clarity.

Material and Methods: This section of material and methods /procedures should be concise but detailed enough to enable the reader to reproduce the experiments / methodology. Commonly used procedures and methods in detail need not be described, but require a reference to the original source.

Results (Including Tables/Fig/Images): Give only brief findings, presented in the form of tables or figures, should be included without duplication of presentation and no discussion of the significance of the data, either tables or figures be given, avoid duplication of data.

Discussion should present the significance of the present data under the prevalent understanding and interpretation of the phenomenon. Speculative discussion is allowed but it should be concise and corroborated by the presented data.

Conclusion: summarizes the study and is drawn from the results and discussion, should not be more than 100 words.

Acknowledgements/ Financial Acknowledgements if any, should be placed at the end of Conclusion before References.

References (In Vancouver Style ie in numbers serially arranged as per standard format).

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1. Lindley, S. T., Estimation of population growth and extinction parameters from noisy data. Ecol. Appl., 2003, 13, 806-813.
2. Martin, H., The Archaean grey gnesisses and the genesis of continental crust. In Archaean Crustal Evolution (ed. Condie, K. C.), Elsevier, Amsterdam, 1994, pp. 205-259.

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Systematic Reviews or Meta-Analysis should be systematic, critical assessments of most recently updated literature and data sources pertaining to basic biological or bio-medical science topics that include a statistical technique for quantitatively combining the results of multiple studies that measure the same outcome into a single pooled investigation. Data must be searched for and selected systematically for inclusion and critically evaluated, and the search and selection process for compiling the review must be mentioned. The text should NOT exceed 5000 words excluding abstract, references, tables and figures.

Each of the sections of the Systematic Review or Meta Analysis articles should include specific sub-sections as follows:

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Screening And Isolation of Lactic Acid Bacteria from the Gut of African Palm Weevil *(Orycete Rhinoceros)* Larvae as Starter Culture in Yoghurt Production

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ABSTRACT:
African palm weevil larvae *(Oryctes rhinoceros)* are widely consumed in the South-South region of Nigeria and valued as a nutritious traditional food. The larvae, naturally distributed across tropical regions, are mostly harvested from the wild. This study investigated the presence and viability of lactic acid bacteria (LAB) in the gut microbiota of the larvae as a potential alternative starter culture for yoghurt production. Larvae were aseptically collected from Odi community, dissected, and cultured using the pour plate technique on de Man Rogosa Sharpe (MRS) agar. Incubated cultures were purified by streaking, and isolates were identified microscopically based on cell morphology, catalase reaction, and Analytical Bacterial Identification System (ABIS) Map. The isolate was used to ferment milk, and key indicators such as pH, titratable acidity, and organoleptic properties were assessed. The LAB isolate from the larvae showed no effective fermentation activity, maintaining a near-neutral pH (6.8), low titratable acidity (72%), fresh-milk odor, and absence of curd formation. In contrast, the standard yoghurt starter culture produced the expected uniform gel structure, characteristic yoghurt aroma, higher titratable acidity (96–120%), and pH 4.5, confirming its functional suitability for yoghurt production. The findings demonstrate that the LAB isolate from *Orycete rhinoceros* larvae is not appropriate as a yoghurt starter culture due to its inability to meet essential fermentation, safety, and physicochemical requirements. Further research should include molecular identification and screening for functional LAB to determine whether safe, technologically relevant strains exist within the larvae microbiome.

KEY WORDS: Yoghurt, Lactic acid bacteria, Starter culture, Palm weevil larvae, *Orycete rhinoceros*, Food safety.

INTRODUCTION

Yoghurt is a very nutritious food and its continued consumption in the Western World owes much to the development of its health food image (Early, 1998).The methods of production of yoghurt have, in essence, changed little over the years and although there have been some refinements, especially in relation to lactic acid bacteria, that bring about the fermentation. Yoghurt is produced in the form of a highly viscous liquid. Yoghurt is also produced in a drinking form and can be frozen or blended with other ingredients to create, for example, mousse type products, sorbet, yoghurt ice-cream or other forms of dairy dessert (Early, 1998). The initial popularity of yoghurt in Western Europe owed much to the work of the Russian and Metchnikoff, 1908 he attributed the good health and longevity of Balkan peasants to the effects of certain bacteria in the yoghurt they consumed. He postulated the theory that prolongation of life would follow ingestion of a lactic acid bacterium named as

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A Review on Polymers Used in Pharmaceutical Formulations

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ABSTRACT:
Pharmaceutical formulations and are therefore an essential part of a pharmaceutical formulation. Their flexibility, having so many chemical structures, physical characteristics, and behaviors, permits tight control of drug release profiles, greater stability, and even better bioavailability. The review has successfully presented the centrality of pharmaceutical science to governments, mainly because of the use of polymers. We consider how polymers are classified on the basis of their source (natural, synthetic, or semisynthetic) and then the role that they play in a formulation, either as binders, disintegrants, coating agents, or matrix formers. We explore the use of polymers in the development of advanced delivery systems such as controlled-release matrices, nanoparticles, micelles, and hydrogels (purposefully designed to avoid obstacles posed by the physiology and deliver targets). Particular focus is on so-called smart or stimuli-sensitive polymers, which can undergo a property change in response to an environmental stimulus such as pH, temperature, or a particular enzyme, resulting in site-specific release of a drug. This abstract relies on the critical analysis of the underlying postulates and modern contributions to polymer-based drug delivery and thus emphasizes the revolution brought to the development of more effective, safer, and patient-friendly therapeutic alternatives through polymer science.

KEY WORDS: Polymers, Pharmaceutical Formulations, Synthetic & Natural Polymers.

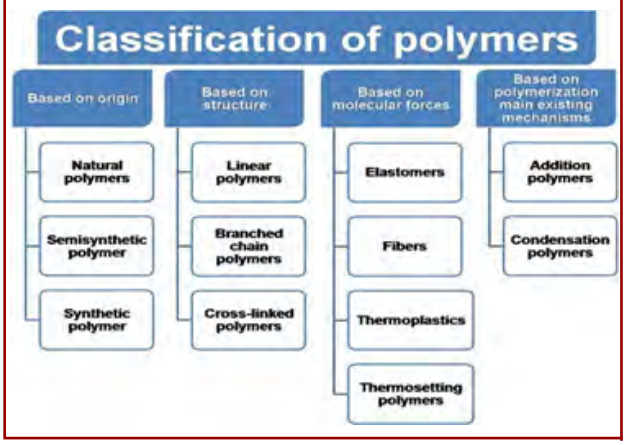
INTRODUCTION

In contemporary pharmaceutical science, the polymers are fundamental ingredients that are indispensible, extending far beyond the more mundane use as inert excipients to form the core of sophisticated drug delivery systems [1,2]. The functionality of such big molecules is carefully designed into formulations so as to regulate release characteristics, stabilize the formulation and curtail the bioavailability of active pharmaceutical ingredients (APIs) [2,3]. Polymers can also be used to modulate the pathway of a drug through the human body to produce a controlled-release dosage form that maintains steady therapeutic levels and thus has reduced the number of doses required and the dose-limiting side effects [3-5]. The use of polymers can be attributed to the capacity to regulate drug release through possible processes such as diffusion, erosion and swelling [5]. Not only does the category of natural polymers such as cellulose and starch adapted as a binder and disintegrant, semisynthetic polymers such as hydroxypropyl methylcellulose (HPMC) in sustained-release matrices and advanced synthetic polymers such as poly (lactic-co-glycolic acid) (PLGA) in biodegradable implants determine

the release pattern of the drug and the therapeutic performance of the drug, but also forms the basis of a huge industry in the pharmaceutical industry [6-10]. New evolutions in polymeric materials, such as the creation of smart polymers that react to certain physiological stimuli, are bringing a new generation of highly-targeted and personalized therapies, and polymers are at the core of the future of pharmaceutical formulation [10].

Classification of Polymers: Polymers [11-13] can have different chemical structures, physical properties, mechanical behavior, thermal characteristics and can be classified in different ways by following below are.

Figure 1: Classification of Polymers



Based on Origin

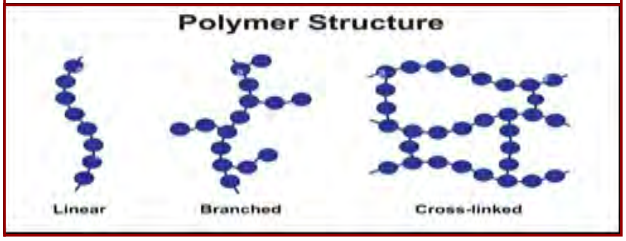
1. Natural Polymers: Natural polymers are those which are derived from either plants or animals. We refer to them as animal and plant polymer.
2. Examples : Cellulose, Jute, Silk, Wool, RNA, DNA, Natural rubber
3. Semisynthetic Polymers: Semisynthetic fibres are created from natural fibres that have been chemically treated to improve specific physical traits, such as tensile strength and lustre.
4. Examples include Cupra, ammonium silk, and viscose rayon.
5. Synthetic Polymers: Synthetic fibres are manufactured in laboratories through the polymerisation of fundamental chemical components [13 & 14].

Examples include nylon, Orlon, polystyrene, PVC, and Teflon.

Based on structure

1. Linear polymers: In these polymer, monomers are interconnected to form an elongated, linear structure. These chains do not possess any additional branches or side chains. Examples: Polyester, Polyethene 2. Branched Polymers: The straight long chain of molecules is accompanied by various side chain. Due to there irregular packing these molecules exhibit low density, tensile strength and melting point [14].
2. Examples: Polypropylene, Amylopectin, Glycogen
3. Crosslinked Polymers: The monomeric units are interconnected to form a three-dimensional framework in which cross link play a crucial role. These cross link contribute to the hardness, rigidity and brittleness of the network structure. Examples: Bakelite, Formaldehyde resin, Vulcanized rubber.

Figure 2: Structure of Polymers



Based on molecular forces

1. Elastomer

These polymers are characterized by polymer chains being held together by the weakest attractive force. They consist of randomly coiled molecular chains with minimal cross links. When a strain is applied, the polymer stretches and upon release of the force, it return to its original position. Such polymers exhibit elasticity and are commonly referred to as elastomers [15]. Examples : Silicone, Natural rubber

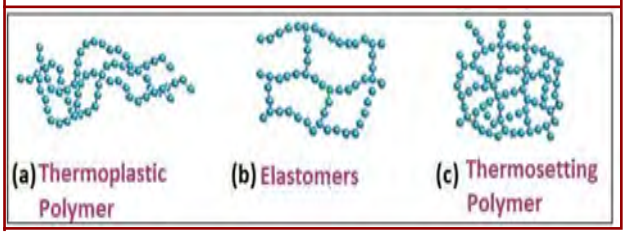
2. Fibers They possess a strong intermolecular attractive force similar to hydrogen bonding. Additionally, they exhibitremarkable tensile strength making them highly valuable in the textile industries.
Examples: Nylon-66, Terlyene

3. Thermoplastic Polymers

Polymers with intermolecular force between elastomer and fibers can be easily shaped by heating and then cooling at room temperature. These polymers may have a linear or branched chain structure. Examples : Acrylic, Polypropylene

4. Thermosetting Polymer This polymer exhibit high hardness and remain non melting when exposed to heat. They do not soften when subjected to pressure and cannot be reshaped. Due to their cross-linked structure these polymers are not recyclable.
Examples:Melamine,Silicone,Polyurea.

Figure 3: Structure of Polymers based on molecular forces



Based on Polymerization Addition Polymer

1. Addition Polymer: Addition polymers are formed by repeatedly adding monomers without removing any byproducts. As a result these polymers consist of all the atom from the monomers making

them a multiple of the monomer unit [15-19]. Examples: Orion, Teflon There are two types of addition polymer:

1. Homopolymer The formation of addition polymers due to the polymerization of single polymeric species is called homopolymer.
2. Copolymer The formation of addition polymer which occur due to addition polymerization from two different monomer is called a copolymer.
3. Condensation Polymer: The formation of these compounds occur through the combination of two monomers resulting in the elimination of small molecules such as water, alcohol, or ammonia. Ester and amide linkage are present in their molecular mass does not correspond to an integral multiple of monomer units [9]. Examples: Polyamide, Polyurethane

Characteristics of an ideal polymer:

1. It should be inert and compatible with the terrain.
2. It should be non- poisonous and physiologically inert.
3. It should be fluently administrable.
4. It should be easy to fabricate and must be affordable.
5. It should have good mechanical strength.
6. It must have comity with utmost of the medicines.
7. It mustn't negatively affect the rate of release of the medicine.
8. It mustn't have tendency to retain in towel and must be a good biodegradable material.
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Mechanism of drug release:

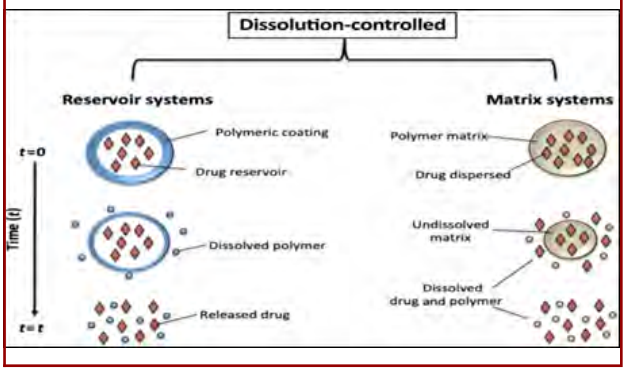
1. Dissolution controlled release.
2. A Matrix dissolution control
3. B. Encapsulation dissolution control
4. 2.Diffusion controlled release.
5. A. Reservoir device
6. B. Matrix device
7. 3.Combination of dissolution and Diffusion release systems
8. 4.Osmotic controlled release
9. 5.Ion exchange system

Dissolution controlled release: Controlled-release drug formulations can be created by reducing their dissolution rate. Strategies to achieve this include the development of suitable salts or derivatives, coating the drug with a slowly dissolving substance, or embedding it within a tablet that contains a slowly dissolving carrier. Various methods can be utilized to create dissolution-controlled systems: One approach involves layering the drug with rate-controlling coatings, allowing for pulsed delivery. If the outermost layer is a rapidly dissolving bolus of the drug, it can quickly establish initial drug levels in the body, followed by subsequent pulsed intervals. Another alternative is to deliver the drug in the form of beads, each featuring coatings of varying thicknesses. Due to the different coating thicknesses, the release of the drug will occur progressively. Beads with thinner coatings will supply the initial dose, while those with thicker coatings will maintain drug levels later on. This principle underpins spansule technology or microencapsulation. The dissolution rate at a steady state is represented by the Noyes-Whitney equation: dC/DA (C-C) dt h, where dC/dt indicates the rate of dissolution, D represents the drug's diffusion coefficient through the pores, h denotes the thickness of the diffusion layer, A is the surface area of the exposed solid, C is the saturated solubility of the drug. Depending on their technical complexity, these systems can be classified into two categories: A. Matrix type B. Encapsulation type [13-18].

A.Matrix dissolution: Matrix dissolution devices are created by compressing the medication with a slowly dissolving carrier to form a tablet. The controlled release is achieved by: 1. Modifying the tablet's porosity 2. Reducing its wettability 3. Allowing it to dissolve at a slower pace. The rate of drug release depends on the dissolution of the polymer. Examples include Dimetane Extencaps and Dimetapp Extentabs.

B. Encapsulation dissolution/reservoir dissolution-controlled system: The microencapsulation method involves coating or enclosing drug particles. These pellets are placed inside hard gelatin capsules, commonly referred to as 'spansules'. Once the coating material dissolves, the entire drug contained within the microcapsule becomes readily available for dissolution and absorption. In this case, the drug release is influenced by both the dissolution rate and the thickness of the polymer membrane, which can vary from 1 to 200 µ. The rate at which the coat dissolves is affected by the stability and thickness of that coating. Examples include: 1. Ornade spansules. 2. Chlorpheniramine repetabs [11-15].

Figure 4: Dissolution model for the Reservoir & Matrix systems for Encapsulation



2. Diffusion controlled release: Diffusion systems are defined by the fact that the rate at which a drug is released depends on its movement through an inert membrane barrier, typically made of an insoluble polymer. Generally, two categories of diffusional systems are identified: reservoir devices and matrix devices. In reservoir devices, the drug release adheres to Fick's first law

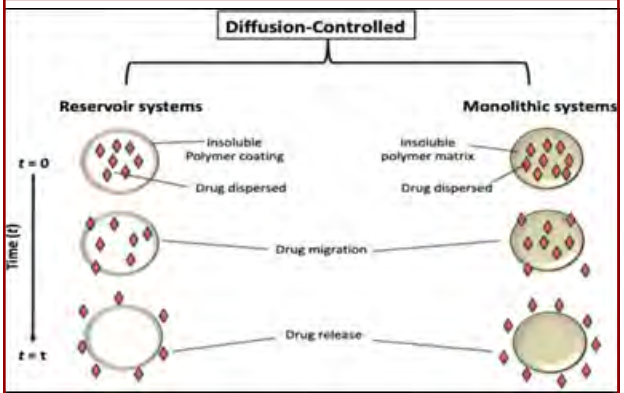
of diffusion. Here, D represents the diffusion coefficient of the drug within the polymer, J denotes the flux (amount per area per time), and dc/dx indicates the change in concentration concerning polymer distance [10-13].

These systems can be classified into two categories: A.Reservior Devices , B.Matrix Devices

A. Reservoir Device: Reservoir devices consist of a drug core, known as the reservoir, which is encased in a polymeric membrane. The characteristics of the membrane influence how quickly the drug is released from the system. One of the benefits of reservoir diffusional systems is that they can achieve zero-order delivery, and the release rate can be adjusted depending on the type of polymer used. However, reservoir diffusional systems have drawbacks, including the need for the system to be physically implanted at the site, challenges in delivering high-molecular-weight substances, and the risk of dangerous dose dumping if the system ruptures [7-9].

B. Matrix Devices/ Monolithic system: A matrix device is made up of a drug that is uniformly distributed within a polymer matrix. In this model, the drug located in the outer layer, which is in contact with the bathing solution, dissolves first and then diffuses out of the matrix. This process continues as the boundary between the bathing solution and the solid drug shifts inward. Clearly, for this system to be governed by diffusion, the dissolution rate of the drug particles within the matrix must significantly exceed the rate at which the dissolved drug diffuses out of the matrix.

Figure 5: Dissolution model for the Reservoir & Matrix systems for Monolithic system



3. Combination of Dissolution and Diffusion release systems: These systems can integrate both the diffusion and dissolution of the drug and the matrix material. The drug can diffuse from the dosage form similar to some previously described matrix systems, while the matrix simultaneously undergoes a dissolution process. The complexity arises from the fact that as the polymer dissolves, the path length for the drug's diffusion may change. This typically results in a moving boundary diffusion scenario. Zero-order release can occur only when surface erosion takes place and the surface area remains constant over time. A key benefit of such a system is that the bioerodible nature of the matrix prevents the formation of a ghost matrix, thus eliminating the need for removal from implant sites. However, the drawbacks of this system include the challenges in controlling release kinetics due to multiple release processes and the potential toxicity of the degraded polymer must be taken into account. Another approach to creating a bioerodible system is by chemically bonding the drug directly to the polymer. Typically, the drug is released from the polymer through hydrolysis or enzymatic reactions. A third type, which employs a combination of diffusion and dissolution, is the swelling-controlled matrix. In this case, the drug is dissolved within the polymer; however, instead of an insoluble or eroding polymer as seen in prior systems, the polymer swells. This swelling allows water to penetrate, leading to drug dissolution and diffusion from the swollen matrix. In these systems, the rate of release is strongly influenced by the polymer's swelling rate, the drug's solubility, and the proportion of the soluble fraction within the matrix. This system often minimizes burst effects since polymer swelling must first occur before drug release [11-18].

4.Osmotic controlled release: In these systems, osmotic pressure acts as the driving force for the controlled release of medication. Imagine a semi-permeable membrane that allows water to pass through but blocks drug molecules. A tablet with a drug core encased by such a membrane, when immersed in water or any bodily fluid, will draw water into the tablet due to the difference in osmotic pressure. These systems usually come in two distinct configurations. The first type features the drug in a solid core along with an electrolyte, which dissolves upon water interaction. The electrolyte creates a significant osmotic pressure difference. The second configuration contains the drug in a solution inside an impermeable membrane within the device, while the electrolyte is situated around the bag. Both types have one or more holes drilled through the membrane that permit drug release. In the first scenario, the high osmotic pressure is alleviated by forcing a solution containing the drug out through the opening. Likewise, in the second scenario, the elevated osmotic pressure compresses the inner membrane, causing the drug to be expelled through the hole. The benefits of osmotically controlled devices include the ability to achieve zero-order release. There is no need for reformulation for various drugs, and drug release is not affected by the surrounding environment of the system. However, the drawbacks of these systems are that they can be significantly more costly than traditional alternatives, and the quality control measures required are more extensive compared to standard tablets [5-15].

5.Ion exchange system: Ion-exchange systems typically utilize resins made of cross-linked, water-insoluble polymers. These polymers feature functional groups that form salts, positioned at regular intervals along the polymer chain. The drug is attached to the resin and is released through an exchange with ions in the vicinity of the ion-exchange sites. The process can be represented as Resin Drug + X → Resin-X + Drug, and conversely, Resin Drug + Y → Resin-Y + Drug, where X and Y are ions found in the gastrointestinal (GI) tract. The released drug then diffuses out from the resin. The drug-resin complex is formed by combining the resin with the drug solution, either through repeated exposure of the resin to the drug in a chromatography column or through

extended contact in a solution. The timing of drug release from the resin is determined by factors such as the diffusion area, length of the diffusion path, and the rigidity of the resin, which relates to the amount of cross-linking agent used during resin production. This system is particularly beneficial for drugs that are prone to degradation due to enzymes, as it provides a protective mechanism by temporarily altering the substrate. However, this controlled release method has the drawback that the release rate depends on the concentration of ions in the administration area. While the ionic concentration in the GI tract generally remains fairly stable, variations in diet, fluid intake, and individual intestinal content can influence the rate at which the drug is released. One enhancement to this system is the application of a hydrophobic rate-limiting polymer, like ethyl cellulose or waxes, as a coating for the ion-exchange resin. These systems depend on the polymer coating to regulate the rate of drug release [3-14].

Application of polymers used in pharmaceutical formulation: Polymers are play a vital role in pharmaceutical formulations given as follows:

Tablets: Tablets are the most prevalent form of dosage for medications intended for oral administration. The drug's release from the tablet can be regulated by modifying the formulation's design and components. In tablet formulations, polymers serve as disintegrants and binders. For example, disintegrants include starch, cellulose, alginates, polyvinylpyrrolidine, and sodium CMC. Binders made from polymers consist of glucose, starch, HPMC, gelatin, alginic acid, polyvinylpyrrolidine, sucrose, and ethyl cellulose. Additionally, polymers can be used to conceal a drug's unpleasant taste and to provide enteric coating for tablets, such as shellac and zein. Microcrystalline cellulose (MCC) improves the compressibility of tablets [2-10].

Capsules: Capsules are typically made from gelatin. There are two types of gelatin: hard gelatin and soft gelatin, each with a different composition. Fillers like microcrystalline cellulose (MCC) and starches are utilized to occupy space within the capsule. To address the issue of aggregation, various polymers such as starch and sodium starch glycolate are combined with the capsule material [2-13].

Polymers in gels: Gel systems are comprised of either physical or chemical cross-links that limit the movement of interconnected polymer chains. Gels exhibit unique rheological characteristics. Cross-linked gels are often referred to as hydrogels. These materials are also classified as smart polymers because they exhibit varying gelling behaviors depending on the water environment. The most frequently utilized hydrogels include poly(hydroxyethyl methacrylate), poly(methacrylic acid), and poly(acrylamide). In the pharmaceutical sector, cross-linked gels are mainly employed for localized drug delivery to the skin, oral cavity, vagina, and rectum [7-19].

Swelling controlled release systems: In numerous drug delivery systems, the size of the dosage form can change during the drug release process due to the swelling of the polymer matrix. While the primary mechanism for drug release is diffusion, examples of systems that demonstrate swelling-controlled release include physically and chemically crosslinked gels. For controlled drug release, chemically crosslinked hydrogels, such as poly(hydroxyethylmethacrylate), have been utilized to facilitate controlled drug release from medical devices, whereas physically crosslinked hydrogels that rely on swelling can be easily produced by directly compressing a drug with a hydrophilic polymer, such as HPMC [8-16].

Temperature-responsive drug release: Numerous studies have been conducted on the design and use of controlled systems for drug delivery that utilize temperature as an external trigger. The polymers employed to achieve such release characteristics are known as thermoresponsive polymeric systems. Typically, homopolymers and copolymers of N-substituted acrylic and methacrylate amides (for example, poly(isopropyl acrylamide)) are used for these applications. More specifically, there are two categories of thermoresponsive polymer systems: those that demonstrate a positive temperature response and those that exhibit a negative temperature response. Polymers in the first category show an upper critical solution temperature, below which the polymer contracts as the temperature decreases. In contrast, negative temperature-dependent polymers possess a lower critical solution temperature and will shrink when the temperature rises above this threshold [6-14].

Polymers in parenteral: We have various kinds of polymers, such as methacrylic acid and its alkyl amide derivative. Methacrylic acid functions as an interferon inducer, which is effective in treating cancer-related illnesses, while its alkyl amide variant serves as a plasma expander, increasing plasma levels in the human body. For instance, insulin injections are utilized in diabetes management, and multiple polymer types are employed in their formulation, acting as reservoirs that bond with insulin and release it at the intended site [4-11].

Ocular drug delivery systems: Enhancing the ocular contact duration of solutions involves adding polymers to an aqueous medium such as polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), methylcellulose, carboxymethylcellulose (CMC), and hydroxypropyl cellulose (HPC). The increased viscosity of the solution helps to minimize its drainage. By elevating the viscosity of a pilocarpine solution from 1 to 100 cps through the addition of methylcellulose, the rate constant for solution drainage was decreased by a factor of ten, while the concentration of pilocarpine in the aqueous humor only increased twofold. Ocusert consists of a drug reservoir, which is a thin disk made of a pilocarpine-alginate complex, situated between two clear discs of microporous membrane created from ethylene-vinyl acetate copolymer. These microporous membranes allow tear fluid to flow into the drug reservoir, facilitating the dissolution of pilocarpine molecules, which are then released at a steady rate of 20 or 40 mg/hr for a duration ranging from one hour to seven days [2-9].

Application in cosmetics: The treatments for hair and skin that are revealed include a novel quaternary chitosan derivative. These

derivatives serve as effective agents, especially for keratin in hair, as well as for enhancing and conditioning hair. Several products are available on the market, such as oxidation hair coloring formulas, hair toning products, skin creams, hair setting lotions, hair treatment solutions, and gels.

Nanoparticles and microparticles: Polymeric nanoparticles and microparticles are utilized for drug encapsulation, offering protection against degradation, regulated release, and targeted delivery. These particles can be engineered to respond to specific physiological conditions, such as changes in pH or temperature, releasing the drug only under predefined circumstances. PLGA nanoparticles are widely researched for delivering anticancer drugs and vaccines, safeguarding the encapsulated drug from degradation and facilitating controlled release. They can also be modified with targeting ligands for precise drug delivery. Like nanoparticles, polymeric microparticles are employed for drug encapsulation and regulated release and are frequently utilized in depot formulations to ensure sustained drug release over longer durations.

The creation of advanced medication delivery systems that enhance patient adherence and treatment effectiveness is significantly reliant on polymers. Targeted delivery systems direct medications to specific tissues or cells, while controlled-release formulations extend the therapeutic effect's duration. Polymeric microparticles and nanoparticles provide protection, regulated release, and targeted administration while adapting to physiological conditions to achieve optimal therapeutic outcomes. Their functionality and versatility make them a vital component of modern medication delivery systems [3-15].

Water-soluble polymers: New applications for water-soluble synthetic polymers span a wide array, including scientific initiatives like drug delivery systems and tissue engineering scaffolds, as well as environmental efforts such as removing heavy metals. Fields focused on information generation also explore fresh opportunities for these materials, such as in electrically responsive or optical films. Water-soluble synthetic polymers have been engineered with properties previously only seen in natural polymers to meet the demands of these innovative applications. The introduction of reactive functional groups is not the only method to address specific challenges. This structural versatility places water-soluble polymers in a pivotal role within the realms of nanotechnology and smart materials.

This brief overview highlights the most recent advancements in the applications of water-soluble synthetic polymers, particularly concentrating on polyethylene glycol, polyvinyl alcohol, polyacrylamide, polyvinylpyrrolidone, and poly (N-isopropyl acrylamide). Through this summary, the intelligent features and sensitive structural control accessible to this class of materials via manipulation of strong hydrophilic interactions will be clarified. Polyethylene glycol, commonly referred to as PEG or polyethylene oxide (PEO) based on its molecular weight, is a typical water-soluble polymer. It has been utilized in a variety of applications, including lubricating coatings, osmotic pressure agents, electrolyte solvents, cosmetic ingredients, and medical laxatives. Recent advancements in nanoscience and technology, as well as in environmental engineering, have opened new opportunities for these polymers and are propelling the development of innovative properties [2-8].

Types of Polymers in Pharmaceutical Drug Delivery

1.Polymers in floating drug delivery system: Polymers play a vital role in floating drug delivery systems, helping ensure that medications reach specific areas of the gastrointestinal tract, particularly the stomach. Researchers have been exploring natural polymers for their effectiveness in targeting drug delivery to the stomach. Some notable examples include chitosan, pectin, xanthan gum, guar gum, gellan gum, karaya gum, psyllium husk, starch, and alginates [3-9].

2.Polymers in Mucoadhesive Drug Delivery Systems: The latest advancements in mucoadhesive polymers are making waves in drug delivery via the buccal route. These innovative polymers provide several advantages, such as adhering longer to the mucosal surface, enhancing penetration through the mucus layer, targeting specific areas, and minimizing enzyme activity. These characteristics make them incredibly valuable for delivering a range of therapeutic agents through the buccal mucosa. Current studies are delving into the use of lectins and "lectinomimetics" to ensure safe and effective drug delivery through this pathway.
3.Polymers for Colon Targeted Drug Delivery: Polymers are essential in colon-targeted drug delivery systems. They safeguard drugs from being degraded or released prematurely in the stomach and small intestine, ensuring that the medication is released in the proximal colon.

For instance, Wong et al. investigated the release of dexamethasone and budesonide from formulations containing guar gum and discovered that drug release significantly increased in simulated colonic fluid when higher concentrations of galactomannanase were present. A new colon-targeted tablet formulation was developed using pectin as a carrier, with diltiazem hydrochloride and indomethacin as model drugs. In vitro tests indicated that these dosage forms released minimal amounts of the drug in the stomach and small intestine, while most of the drug was released in the colon. Additionally, McLeod et al. synthesized glucocorticoid-dextran conjugates, incorporating dexamethasone and methylprednisolone for enhanced delivery.

Advantages of polymer used in pharmaceutical formulation

1. Colloidal drug carrier systems that utilize polymers made of small particles offer significant benefits in drug delivery due to their enhanced drug loading and release capabilities.
2. In controlled drug delivery, a polymer (either natural or synthetic) is combined with a drug, facilitating an effective and regulated dosage while preventing overdose.
3. Degradable polymers break down into biologically compatible molecules that can be absorbed and eliminated by the body through normal pathways.
4. Reservoir-based polymers provide multiple advantages,

such as improving the solubility of poorly soluble drugs and minimizing adverse side effects.

5. Magneto-optical nanoparticles that are polymer-coated and targeted are detectable by both optical and MRI methods, whereas quantum dots are only detectable optically.
6. Some quantum dots contain calcium, which is recognized as toxic to humans. In contrast, polymer-coated or targeted magneto/optical nanoparticles consist of iron oxides/ polymers that are considered safe, indicating a promising future.
7. Dextran, a common polymer used for coating iron oxide, has been utilized as a plasma expander and has affinity for iron, and it has been employed in treating iron-deficiency anemia since the 1960s, still continuing today.
8. In controlled release applications, certain polymers, such as polyurethanes known for elasticity and polysiloxanes valued for insulating properties, are chosen for their specific non-biological physical attributes.
9. Modern polymers like poly 2-hydroxyethyl methacrylate, polyvinyl alcohol, and polyethylene glycol are preferred due to their inert qualities and the absence of leachable impurities.
10. Biodegradable polymers are biocompatible, ensuring that no residual dosage remains at any time, while maintaining their properties until the drug is fully depleted.
11. In hydrogels used for drug delivery, the characteristics of polymer materials like PEG (a commonly utilized polymer in designing hydrogels) can be adjusted to optimize features like pore size, thereby controlling the diffusion rate of the delivered drugs. PEGylation has been found beneficial for various conditions, including hepatitis B and C, cancer-related neutropenia (PEG-GCSF), and several cancer types [PEG]. Glutaminase was combined with the glutamine anti-metabolite 6-diazo-5-oxo-norleucine (DON).
12. Polymers serve a variety of roles, from acting as films or binders in tablet coatings to flow-managing agents in liquids or emulsions, enhancing drug safety and modifying delivery properties. Micelles, due to their smaller size, have a brief circulation time in the body, granting them an advantage in easily penetrating tumor cells through the EPR effect.

Disadvantages of polymer used in pharmaceutical formulation

1. It cannot handle very high temperatures because all plastics start melting quickly compared to essence.
2. The strength-to-size ratio of polymer is lower, whereas for essence it is higher.
3. It is difficult to machine smoothly, and the machining speed is limited.
4. The heat capacity of polymer is much lower, making it unsuitable for heat-related applications.
5. It is not possible to create heavy structures with polymer because its structural strength is much lower.
6. Disposal becomes a problem because some polymers cannot be recycled, while all essence can be reclaimed.
7. After being delivered, polymers have a propensity to release a significant amount of medication rapidly.
8. From the beginning, polymers exhibit a high degree of drug release.

CONCLUSION

To sum up, the use of polymers has made a big difference in the pharmaceutical industry, starting from ancient times. Polymers have special qualities that make them perfect for many uses in medicine. They are commonly used in everyday medicine forms like tablets and capsules as binders, fillers, dissolving agents, and coatings. These help keep the medicine strong, uniform, and make sure the active ingredients are released at the right time. In special medicine forms that control how fast a drug is released, both types of polymers—those that don't break down and those that do—are important. They help manage how much drug is released and when, which makes treatment more effective. Polymers are also widely used in medicine packaging because they are flexible, strong, and protect against chemicals and bad weather. Using polymers in the pharmaceutical industry helps make medicines work better, last longer, and be safer. New discoveries in polymer science could lead to even more useful ideas and uses in medicine.

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Effects of Exposure to Magnetic Fields in MRI: A Review of Current Evidence and Future Directions

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ABSTRACT:
This review aims to examine the biological effects of Magnetic Resonance Imaging (MRI) exposure in healthcare professionals working with high-field MRI systems (1.5 Tesla and above). By synthesizing current evidence, it seeks to inform future safety guidelines and highlight potential risks associated with cumulative, long-term exposure among MRI technicians and radiologists.A systematic literature search was conducted on studies published from 2000 to 2024. Databases (e.g., PubMed, Scopus) were queried using key terms such as “MRI exposure,” “occupational health,” and “high-field MRI.” Studies were evaluated based on methodological rigor, relevance to occupational settings, and reported biological effects. The search aimed to identify both short-term and long-term physiological impacts of repeated MRI exposure. Although short-term MRI exposure is generally safe, healthcare workers consistently operating high-field MRI systems may experience transient sensory disturbances (e.g., vertigo, nausea) and possible long-term physiological effects. Findings suggest an elevated concern for cumulative exposure, yet conclusive evidence on the magnitude and nature of these risks remains limited due to variability in study designs and outcomes. While MRI technology plays a pivotal role in diagnostic medicine, ongoing research is needed to clarify the occupational hazards for healthcare professionals exposed to high-field environments. Improved monitoring, standardized safety protocols, and comprehensive longitudinal studies are recommended to ensure the continued safe use of MRI in clinical settings.

KEY WORDS: electromagnetic fields; Magnetic Resonance Imaging; MRI safety.

INTRODUCTION

Magnetic Resonance Imaging (MRI) is a cornerstone of modern diagnostic medicine, providing high-resolution images of internal body structures using non-ionizing electromagnetic fields [1-3]. Since its introduction in the 1980s [4], MRI has revolutionized the way medical professionals diagnose and monitor a wide range of conditions, from neurological disorders to cardiovascular diseases. The widespread use of MRI is due in large part to its ability to offer detailed anatomical and functional information without the risks associated with ionizing radiation, such as that used in X-rays and CT scans. This has established MRI as a preferred modality in many clinical and research settings [5-8].

However, as MRI technology has advanced, so too have concerns regarding its long-term safety, particularly for healthcare workers who are regularly exposed to the powerful magnetic fields generated by MRI machines. While MRI is

generally regarded as safe for patients due to the relatively short duration of exposure, the same may not hold true for medical staff, who may be exposed to these fields for hours each day over the course of their careers. These concerns have been amplified with the increasing use of high-field MRI systems, such as those operating at 3 Tesla (T) or higher, which offer greater image resolution and contrast but also produce stronger magnetic fields that could pose a greater risk of biological effects [9,10].

The potential biological effects of MRI exposure are associated with three primary sources: static magnetic fields, time-varying gradient magnetic fields, and RF fields [11]. Static magnetic fields, which are a constant feature of MRI, can interact with biological tissues, leading to effects such as magneto hydrodynamic phenomena, which can cause dizziness and other sensory disturbances [12,13]. Time-varying gradient magnetic fields are used to spatially encode the MRI signal and can induce electrical currents in the body, potentially leading to peripheral nerve stimulation. RF fields are responsible for heating tissues, and while regulations limit the specific absorption rate (SAR) to prevent excessive heating, higher field strengths could pose a greater risk of localized tissue damage [14].

A key concern for healthcare workers is the cumulative effect of prolonged exposure to these fields over time. While individual MRI examinations may not pose a significant risk, repeated exposure over months or years could potentially lead to adverse health outcomes. For example, studies have reported transient effects such as dizziness, vertigo, metallic taste, and concentration difficulties among MRI technicians and radiologists, suggesting that there may be physiological consequences to long-term occupational exposure to MRI environments [15-17].

To date, research on the long-term effects of MRI exposure has been inconclusive. Several studies have examined the acute effects of MRI exposure, often focusing on short-term symptoms such as dizziness or nausea. However, fewer studies have investigated the long-term health consequences for healthcare workers who are exposed to MRI environments on a regular basis. Existing studies are often limited by small sample sizes, short follow-up periods, or a lack of consistent exposure measurement. Additionally, there is significant variability in the levels of exposure experienced by MRI staff, depending on factors such as their proximity to the MRI machine, the duration of exposure, and the strength of the magnetic field. [18-23].

Despite the growing body of research, many questions remain unanswered. For instance, it is unclear whether chronic exposure to high-field MRI could lead to more serious health conditions, such as cognitive impairment, cardiovascular issues, or even cancer. Additionally, the variability in exposure levels among MRI staff highlights the need for more precise monitoring and risk assessment protocols. Understanding the mechanisms by which MRI exposure might affect biological tissues is crucial for developing effective safety guidelines and minimizing risks for healthcare workers.

Given the increasing reliance on high-field MRI in both clinical and research settings, it is critical to assess the safety of long-term exposure to MRI environments, particularly for healthcare professionals who may be at higher risk. This study aims to provide a comprehensive review of the current literature on the biological effects of MRI exposure, with a particular focus on healthcare workers who are regularly exposed to high-field MRI. By synthesizing existing research, this review will identify both the potential benefits and risks associated with long-term MRI exposure, and will highlight areas where further research is needed to fill existing knowledge gaps. The outome of this review will contribute to the development of more robust safety guidelines for healthcare workers, ensuring that the benefits of MRI technology can be fully realized without compromising the health and safety of those who operate these systems daily. Furthermore, by identifying gaps in the current literature, this study will help to inform future research priorities and guide the development of new safety protocols for high-field MRI environments.

MATERIAL AND METHODS

Figure 1 illustrates a flow chart describing that A comprehensive review was conducted of the biological effects of MRI exposure by systematically searching relevant literature across multiple databases, including PubMed, Google Scholar, Scopus, and Science Direct, ensuring broad coverage of scientific publications and clinical studies .The search was performed using specific keywords and Boolean operators, such as “MRI exposure,” “biological effects of MRI,” “occupational health MRI staff,” “high-field MRI safety,” and “MRI-induced cognitive effects.” Boolean operators (e.g., AND, OR) were applied to refine and combine search terms where necessary. The complete list of all search terms and combinations used in this review is provided in Appendix A to facilitate reproducibility and to support future related research. The search spanned studies published between 2000 and 2024, focusing on research related to MRI machines operating at field strengths of 1.5 Tesla and above.

This threshold was chosen because these field strengths are the most widely used in clinical environments and generate stronger magnetic fields, making them more relevant for evaluating occupational safety. Figure 2 illustrates the study review criteria applied in this review, including both inclusion and exclusion parameters. Studies investigating biological effects on healthcare staff—such as MRI technicians, radiologists, and other medical professionals frequently exposed to MRI environments—were included.

In addition to the database search, we conducted a manual review, which involved screening the reference lists of all included studies to identify additional relevant articles

not captured during the initial search. These additional articles were retrieved, read in full, and assessed against the same inclusion and exclusion criteria to ensure eligibility. physiological changes, and potential long-term risks such as DNA damage. Studies that did not meet these criteria were excluded from the review.

Figure 1: Methodology Flow Chart

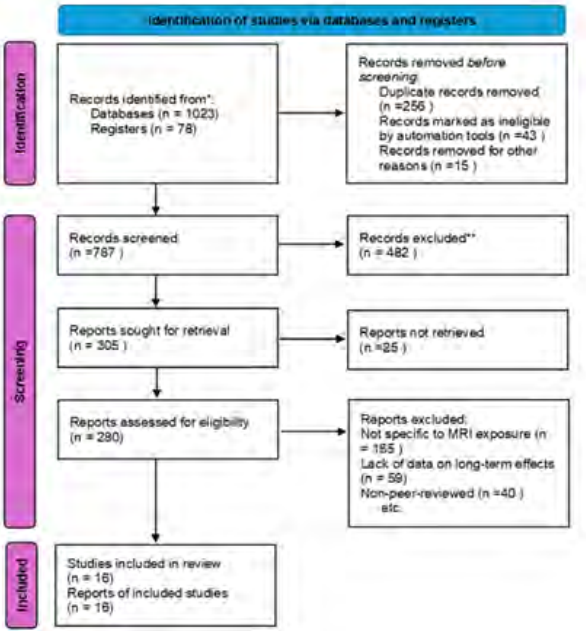

Figure 2. Study Review Criteria

RESULTS

The analysis of studies [24–39] on magnetic resonance imaging (MRI) exposure reveals both immediate and potential long-term health effects for individuals in MRI environments. Studies such as [24] identified short-term subjective symptoms (e.g., drowsiness, concentration issues) among healthcare professionals exposed to MRI. However, symptoms typically resolved within weeks, suggesting possible adaptation. Studies [25–28] explored the physiological impacts of ultra-high-field (UHF) MRI systems (≥7T), noting transient effects like dizziness and vertigo but insufficient evidence for lasting biological damage, underscoring the need for regular monitoring and strict adherence to occupational safety protocols.

For MRI technicians and workers in industries with static magnetic fields, studies [29,30] noted sleep disturbances and inconclusive cancer risks, emphasizing the need for improved exposure assessment and ongoing health monitoring. In particular, the lack of conclusive epidemiological studies limits understanding of MRI's cumulative effects over extended periods, especially at high field strengths. Studies focusing on childhood leukemia and environmental EMF exposure, such as studies [31–39], present conflicting findings. While some meta-analyses [35,39] indicate a potential link between extremely low-frequency (ELF) magnetic fields (≥0.4 μT) and childhood leukemia, others report no definitive associations, suggesting that results may vary by exposure intensity and environmental factors. These discrepancies highlight the necessity for further large-scale, longitudinal studies to clarify long-term EMF impacts, particularly among children and other vulnerable groups.

DISCUSSION

This review highlights the current understanding and gaps in knowledge regarding the health impacts of MRI and EMF exposure. Short-term symptoms like dizziness, concentration issues, and drowsiness are frequently reported by healthcare workers in MRI environments, with most symptoms resolving over time. However, there remains limited and inconclusive data on the long-term effects of high-field MRI and EMF exposure, particularly concerning cumulative health risks such as cancer, reproductive effects, and neurological impacts. Notably, studies focusing on extremely low-frequency (ELF) magnetic fields in residential settings indicate a potential association with an increased risk of childhood leukemia, though findings vary across studies and depend on factors such as exposure intensity and environmental context. The lack of definitive evidence on long-term effects points to an urgent need for more rigorous epidemiological studies, particularly longitudinal research that examines cumulative exposure in both occupational and residential contexts.

For healthcare professionals, especially MRI technicians and radiologists, the growing use of ultra-high-field (≥7T) MRI technology warrants strict adherence to occupational safety protocols, including regular monitoring and exposure assessments. Furthermore, the potential vulnerability of children and other high-risk groups to prolonged EMF exposure emphasizes the need for precautionary measures in both clinical and residential environments. Considering these findings, developing robust, evidence-based guidelines for MRI and EMF safety is essential to balance the benefits of MRI technology with the health and safety of both patients and healthcare workers. Future research should prioritize understanding the biological mechanisms behind EMF-

related health effects and evaluating the long-term impacts on diverse populations to establish clear and actionable safety standards. This study underscores the necessity of proactive safety protocols and continued research to support the safe and effective use of MRI in healthcare settings.

Summary table MRI exposure

Author/Year	Population	MRI Field Strength	Exposure Duration/Type	Reported Symptoms/Effects	Short/Long Term	Severity/Frequency
Zanotti et al., 2015 [24]	MRI technicians	1.5T–3T	Routine occupational work	Drowsiness, concentration issues	Short	Moderate, resolved in weeks
Ladd et al., 2018 [25]	Healthy volunteers	≥7T	Single and repeated scans	Dizziness, vertigo	Short	Mild, transient
Schenck, 2000 [26]	General MRI population	≥1.5T	Routine scanning	Safety and static field interaction effects	Short/Long	Varied, inconclusive for long-term
Huss et al., 2021 [29]	MRI technicians	1.5T–3T	Occupational, routine work	Sleep disturbances	Long	Persistent in subset
Feychting, 2005 [30]	MRI workers	Mixed	Occupational	Inconclusive cancer risk	Long	Not quantified
Schüz, 2011 [35]	Children (environmental EMF)	Residential ≥0.4 μT	Residential exposure	Potential link to childhood leukemia	Long	Risk varies by exposure level
Ghahremani et al., 2020 [39]	Children (environmental EMF)	Residential ≥0.4 μT	Residential exposure	Association with childhood leukemia (meta-analysis)	Long	Risk varies by exposure level

CONCLUSION

This review highlights the current understanding and remaining gaps regarding the health impacts of MRI and EMF exposure in occupational settings. Short-term effects such as dizziness, vertigo, and concentration difficulties are commonly reported but generally resolved over time, while evidence for long-term risks remains limited and inconclusive. The growing use of ultra-high-field MRI underscores the need for rigorous exposure monitoring, standardized safety protocols, and targeted research particularly large-scale longitudinal studies to clarify potential cumulative effects. For now, strict adherence to occupational safety measures remains the most effective strategy to protect MRI staff and patients while enabling the continued safe use of MRI technology.

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Effect of *Mussaenda glabrata* Leaf Extract on Cisplatin Induced Hematotoxicity, Nephrotoxicity And Neurotoxicity in Albino Wistar Rats

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ABSTRACT:
This research was aimed to explore effect of *Mussaenda glabrata* extract of leaves against cisplatin induced hematotoxicity, neurotoxicity and nephrotoxicity in experimental rats. Toxicities were induced in healthy adult wistar rats by administering cisplatin, single dose of 8mg/kg, on 5th day through intraperitoneal route. After completion of nine days dosing with the test drug, on 10th day, various hematological, biomarkers, antioxidants and histopathological studies were performed. *Mussaenda glabrata* leaf extract had a significant beneficial role in cisplatin induced hematotoxicity by increasing the hematological parameters compared to cisplatin treated group. It also exhibited a significant protection against cisplatin induced nephrotoxicity and neurotoxicity by suppressing the biomarkers and by elevating the antioxidant parameters at different doses compared to cisplatin treated group. According to research findings, *Mussaenda glabrata* extract of leaves may be protective against the neurotoxicity, nephrotoxicity and hematotoxicity caused by the anti-cancer medication cisplatin. MGLE's antioxidant action may thus be directly linked to its protection. These results support the theory that antioxidant-rich medicinal herbs may have protective properties.

KEY WORDS: Cisplatin, *Mussaenda glabrata* leaf extract, Hematoprotective, Neuroprotective, Nephroprotective.

INTRODUCTION

Cisplatin is considered one of the key chemotherapy agents for cancer treatment that may be used to treat a variety of human cancers in various organs [1].

Nephrotoxicity can develop from cisplatin buildup in the tubules that are proximal inside the kidney once it is excreted by the kidneys. Subjects having a creatinine clearance more than 60 milliliters per minute are often the only ones who can use it; nonetheless, cisplatin-induced nephrotoxicity is frequent and can restrict dosage and/or dose intensity. When patients who have received a complete course of chemotherapy develop peripheral neuropathy, cisplatin neurotoxicity is medically obvious and may impact the treatment plan and the patient's quality of life [2].

One of the main causes of neurotoxicity is believed to be the build-up of cisplatin within dorsal root ganglia neurons as platinum-DNA adducts. There are currently no medications that can stop the development of cisplatin-induced neurotoxicity and the high frequency of neurotoxicity restricts the chemotherapeutic efficacy of cisplatin [3].

The development of synthetic medications has led to the necessity for alternative therapies and medical care due to their negative effects. From ancient times to the present, herbs and herbal treatments have been used extensively to heal illnesses [4].

Cisplatin's anticancer properties have been attributed to its capacity to bind DNA and generate covalent cross-links, which in turn block transcription and DNA replication. However, the host's development of numerous adverse effects and/or the cancer cells' acquisition of drug resistance limit the complete therapeutic efficacy of cisplatin [5].

Mussaenda glabrata syn. Mussaenda frondosa, also called "Vellaiilai" in Tamil, is a member of the Rubiaceae family and one of the medicinally significant plants. Jaundice, hyperacidity, ulcers, diuretic, wound, leprosy, swelling, asthma, antibacterial, hypolipidemic effect, hepatoprotective action, fever and cough are among the traditional ailments that leaves are used to cure [6].

Till now no study has been reported, regarding, protective effect of *Mussaenda glabrata* leaf extract [MGLE] against cisplatin induced hematotoxicity, nephrotoxicity and neurotoxicity. Hence the current study was designed to demonstrate the impact of extract of leaves from *Mussaenda glabrata* in different doses against cisplatin induced hematotoxicity, nephrotoxicity and neurotoxicity.

MATERIAL AND METHODS

Chemicals: Cisplatin was obtained from Cipla, Mumbai, India. Biochemical estimation kits were obtained from Precision Biomed Pvt. Ltd., Mumbai, India. All chemicals, solvents used for this study were of the analytical grade obtained from reputed suppliers from India.

Animals: Both sexes of experimental rats weighing between 175 and 250 grams were kept in an adequately conditioned animal housing with a 12:12 light-dark cycle at 25 ° ± 5 °C and 50± 5% relative humidity. Every rat was given a commercially accessible standard pellet diet and unlimited access to water. In accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals' (CPCSEA) recommendations, the animals were kept and research was carried out. Institutional Animal Ethics Committee [IAEC] approved the study bearing Reference No.: SDCP/IAEC/13/2020 dated 15/02/2020.

Preparation of *Mussaenda Glabrata* leaf extract [MGLE] [7] Kerala was the source of M.glabrata. The plant material was identified by the Department of Botany, University College, Mangalore, 575001. Leaves that had been shade-dried were ground into a reasonably coarse powder. The Soxhlet extractor was used to obtain petroleum ether, chloroform, alcohol, and aqueous extracts of leaves. Till the solvent in the thimble was clear, the extractions were carried out 12 times. The deep brown semisolid substance was stored for later use in an airtight container once the solvent had evaporated.

Phytochemical estimation of the extract [8]: The presence of several phytochemical elements, including alkaloids, proteins, carbohydrates, glycosides, phytosteroles, saponins, tannins and flavonoids were examined by qualitative analysis of the MGLE extract.

Experimental Protocol

Dose selection [9,10]: Cisplatin dosages for rats have been determined to be 200 mg/kg and 400 mg/kg by oral route based on earlier studies of the literature.

Cisplatin induced toxicity model [9,10]: The current study has nine-day duration. On the fifth day, a single intraperitoneal (i.p.) dosage of 8 mg per kg of cisplatin was given to healthy adult Wistar rats in order to cause hematotoxicity, nephrotoxicity, and neurotoxicity. The MGLE was given orally to each group for nine days.

Following nine days of test medication dosage, the rats being studied was put to sleep by ether inhalation on the tenth day (six days following cisplatin injection). From each rat, blood was taken and split into two samples. Also kidneys and brain were removed and one sample was used to estimate hematological parameters while the other was utilized to estimate biomarkers. The kidney's two sections were employed for histological research and antioxidant assessment, respectively. The animal's brain was removed, cleaned in cold saline, blotted and prepared for biochemical along with histological analysis in order to assess neurotoxicity.

Groupings: Good health mature wistar rats were split into four distinct groups of six rats each. Group I: Normal (saline 10 ml/kg oral) for 9 days. Group II: Cisplatin (8mg/kg intraperitoneal) on the 5th day of treatment. Group III: Low dose MGLE (200 mg/kg intraperitoneal) + CIS on the 5th day of treatment. Group IV: High dose MGLE (500 mg/kg intraperitoneal) + CIS on the 5th day of treatment.

RESULTS

Isolation of *Mussaenda Glabrata* Leaf Extract: The practical yield of MGLE from 100g of dried plant powder of M. glabrata by maceration process was found to be 35 g. The evidence of carbohydrates, steroids, triterpenoids, glycosides and flavonoids were confirmed by preliminary qualitative analysis of the MGLE extract.

Effect of MGLE on CIS induced hematotoxicity: MGLE showed high efficacy against CIS induced hematotoxicity. CIS treated rats explored extremely significant ($P<0.001$) decrease in RBC (red blood corpuscles) compared to normal group. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed extremely significant ($P<0.001$) increase in RBC compared to CIS group. CIS treated rats showed

extremely significant (P<0.001) increase in WBC (white blood corpuscles) compared to normal group. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed extremely significant (P<0.001) decrease in WBC compared to CIS group.CIS treated rats showed most significant (P < 0.01) decrease in Hb (haemoglobin) compared to normal group. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed most significant (P<0.001) increase in Hb compared to CIS group.CIS treated rats showed extremely significant (P<0.001) decrease in platelets compared to normal group. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed extremely significant (P<0.001) increase in platelets compared to CIS group. See table 1.

Table 1. Effect of MGLE on CIS induced hematotoxicity

TREATMENT	RBC (106 cell/µL)	WBC (103 cell/µL)	Hb (g/dL)	Platelets (10^5 cell/µL)
NORMAL	4.69±0.101	4.656±0.003	13.825±0.006	6.256±0.009
CIS	2.548±0.073###	7.305±0.067###	10.345±0.007###	2.915±0.001###
MGLE200+CIS	2.913±0.005**###	6.631±0.192**###	10.685±0.087**###	3.391±0.151*###
MGLE400+CIS	3.31±0.026***###	6.039±0.166***###	10.896±0.077***###	3.626±0.171**###

n=6; data are presented as MEAN ± SEM. Statistical significance was determined using one-way ANOVA then Tukey-Kramer multiple comparison test. **P<0.01 and ***P<0.001 indicate significant differences compared to the control group, while ##P<0.01 and ###P<0.001 denote significant differences compared to the normal group.

Effect of MGLE on CIS induced nephrotoxicity serum biomarkers: In this model of experimentation, the CIS induced rats showed an extremely significant (P<0.001) increase in creatinine level when compared with normal control. Prior treatment of MGLE (200 mg/kg) and MGLE (400 mg/kg) showed highly significant (P<0.001) decrease in serum creatinine level respectively when compared with the control group.In this experimental model, the CIS induced rats control showed an extremely significant (P<0.001) increase in urea level when compared with normal control. Prior treatment of MGLE (200 mg/kg) and MGLE (400 mg/kg) showed extremely significant (P<0.001) decrease in urea level respectively when compared with the control group. In this model of experimentation, the CIS induced rats revealed an extremely significant (P<0.001) increase in uric acid level when compared with normal control. Prior treatment of MGLE (200 mg/kg) and MGLE (400 mg/kg) showed more significant (P<0.001) decrease in uric acid level respectively when compared with the control group. See table 2.

Table 2. Effect of MGLE on CIS induced nephrotoxicity serum biomarkers

TREATMENT	CREATININE (mg/dl)	UREA (mg/ml)	URICACID (mg/ml)
NORMAL	0.963±0.009	37.858±0.032	1.587±0.021
CIS	4.457±0.019###	70.808±0.048###	6.312±0.029###
MGLE200+CIS	3.510±0.225**###	59.352±4.008*###	5.065±0.269*###
MGLE400+CIS	3.203±0.297***###	57.809±3.788*###	4.732±0.494**###

n=6; data are presented as MEAN ± SEM. Statistical significance was determined using one-way ANOVA then Tukey-Kramer multiple comparison test. **P<0.01 and ***P<0.001 indicate significant differences compared to the control group, while ##P<0.01 and ###P<0.001 denote significant differences compared to the normal group.

Effect of MGLE on CIS induced nephrotoxicity antioxidants: Investigation of antioxidants in homogenate kidney tissue showed an extremely significant (P<0.001) decrease in GSH compared to normal group. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed more significant (P<0.001) increase in GSH compared to CIS group. Investigation of antioxidants in homogenate kidney tissue showed more significant (P<0.001) decrease in catalase compared to normal group. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed extremely significant (P<0.001) increase in catalase compared to CIS group. Investigation of antioxidants in homogenate kidney

tissue showed more significant (P<0.001) decrease in SOD (super oxide dismutase) compared to normal group. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed extremely significant (P<0.001) increase in SOD compared to CIS group. See Table 3.

Table 3. Effect of MGLE on antioxidants in CIS induced nephrotoxicity antioxidants

TREATMENT	GSH (ηM/g wet gland)	SOD (U/mg wet gland)	Catalase (U/mg wet gland)
NORMAL	17.509±0.058	96.262±0.007	22.75±0.012
CIS	8.737±0.006###	49.566±0.007###	12.08±0.017###
MGLE200+CIS	10.36±0.530*###	66.977±4.388*###	15.729±0.660**###
MGLE400+CIS	10.867±0.548**###	75.715±6.885**##	17.488±1.003***###

n=6; data are presented as MEAN ± SEM. Statistical significance was determined using one-way ANOVA then Tukey-Kramer multiple comparison test. **P<0.01 and ***P<0.001 indicate significant differences compared to the control group, while ##P<0.01 and ###P<0.001 denote significant differences compared to the normal group.

Effect of MGLE on CIS induce neurotoxicity antioxidants: Investigation of antioxidants in homogenate brain tissue showed an extremely significant (P<0.001) decrease in GSH of CIS treated group compared to normal. The rats pretreated with MGLE (200mg/kg) and MGLE (400mg/kg) showed extremely significant (P<0.001) increase in GSH compared to cisplatin control. Investigation of antioxidants in homogenate brain tissue showed an extremely significant (P<0.001) decrease in catalase of CIS treated group compared to normal. The rats pretreated with MGLE (200mg/kg) and MGLE (400mg/kg) showed extremely significant (P<0.001) increase in Catalase compared to cisplatin control. Investigation of antioxidants in homogenate brain tissue showed an extremely significant (P<0.001) decrease in SOD of CIS treated group compared to normal control. The rats pretreated with MGLE (200 mg/kg) and MGLE (400 mg/kg) showed extremely significant (P<0.001) increase in SOD compared to Cisplatin control. See Table 4.

Table 4. Effect of MGLE on CIS induced neurotoxicity antioxidants

TREATMENT	GSH (mmol/g tissue)	SOD (U/g tissue)	CAT (U/g tissue)
NORMAL	17.781±0.024	15.86±0.025	17.735±0.007
CIS	8.715±0.024###	8.558±0.019###	10.556±0.014###
MGLE200+CIS	9.853±0.270**###	10.655±0.489**###	11.648±0.286**###
MGLE400+CIS	10.253±0.360***###	11.490±0.508***###	12.597±0.318***###

n=6; data are presented as MEAN ± SEM. Statistical significance was determined using one-way ANOVA then Tukey-Kramer multiple comparison test. **P<0.01 and ***P<0.001 indicate significant differences compared to the control group, while ##P<0.01 and ###P<0.001 denote significant differences compared to the normal group.

DISCUSSION

This study aimed to determine if *Mussaenda glabrata* extract of leaves could prevent cisplatin-induced hematoxicity, nephrotoxicity, and neurotoxicity in albino wistar rats. The study found that *Mussaenda glabrata* leaf extract significantly reduced the effects of cisplatin. The Rubiaceae family includes the flowering plant species M. glabrata. The entire wild Mussaenda plant has anti-inflammatory properties and is used to treat pruritis, bronchitis, fever, wounds, ulcers, cough and jaundice [11].

One well-known chemotherapeutic medication is CIS. Numerous human malignancies, including as those of thelung, ovaries, bladder, head and testicles have been treated with it. Nephrotoxicity is the most prevalent of the roughly 40 distinct toxicities of cisplatin. Ototoxicity, haematological neurotoxicity, hepatotoxicity, gastrointestinal and cardiotoxicity are additional frequent adverse effects [12,13]. One of the most critical metrics for determining the toxicity of the anti-cancer medication cisplatin in both people and animals is the haematopoietic system. According to the current study, rats treated with CIS for five days experienced considerable toxic effects in their haematological

parameters, while animals pretreated with MGLE shown a strong defence against hematotoxicity. The substantial reduction in the number of erythrocytes, hemoglobin, and platelets validated the harmful effects of CIS on haematological parameters [14].

The CIS demonstrated a significant decrease in the RBC level in the current investigation, confirming the hematotoxicity. When compared to a normal control group, the observed decline was highly significant, and the magnitude of toxicity was quite high. Several factors could be responsible, including bone marrow cell deterioration or an increase in the osmotic brittleness of red blood cells. Therefore, CIS poisoning may result in a reduction of red blood cells due to either haematopoietic tissue activity suppression, erythropoiesis impairment, or both. Because of the increased permeability of the RBC membrane, cisplatin treatment accelerated the destruction of RBCs and decreased erythropoietin, which is a haemopoietic growth factor. This, in turn, caused changes in haematological parameters [14].

In platelets and lymphocytes, CIS induces oxidative stress that may impact their lifespan, trigger apoptosis, and ultimately decrease the quantity of these cells in the circulation. In addition to a drop in RBC count, a decrease in platelet count may result from cisplatin's inhibition of bone marrow function, reduced platelet generation or consumption, or enhanced platelet aggregation. Also results suggests that thrombocytopenia with leukopenia in the cisplatin-treated group may have been caused by the apoptotic impact of cisplatin on platelets and lymphocytes, which in turn decreased the quantity of these cells in the blood. Additionally, decreased haemoglobin and erythrocyte counts may be caused by bleeding from intestinal affections caused by cisplatin and red cell destruction caused by free radicals [14].

Animals treated with CIS in the current study had higher WBC levels. This may be the result of an inflammatory response or the infection brought on by the injection of CIS. Additionally, CIS may cause oxidative stress in lymphocytes and thrombocytes of human being, resulting in their necrosis 14. Due to haemoglobin's sensitivity to oxidative stress, we observed a decrease in platelets and haemoglobin levels following CIS administration. Extracellular signal-regulated protein kinase (ERK) in platelets is activated in a dose-dependent manner by cisplatin, resulting in platelet death and decreased platelet function, both of which are signs of haematological toxicity. RBC, Hb, and platelet counts are beneficially increased in response to MGLE dose-dependent prophylactic therapy against CIS-induced hematotoxicity indicators. An increase in these erythrocytes may be linked to either preventing bone marrow suppression or promoting erythropoiesis. However, the inhibition of ERK in platelets is the cause of the increase in platelet counts. A lower WBC count may be associated with reduced inflammation across the haemopoietic system [14].

The CIS-treated group exhibits a substantial increase in urea, creatinine and uric acid in nephrotoxicity. The two primary indicators of nephrotoxicity and hepatorenal development are serum creatinine and urea. The natural product of muscle digestion, serum creatinine is removed unaltered by renal system. An anomaly in the kidney's glomerular filtration mechanism might be the cause of the rise in blood creatinine levels. The waste product produced by the liver's urea cycle during protein metabolism is called urea. Serum urea levels rise as a result of GFR deficit and decreased blood volume. The enzyme uridase produces uric acid as a byproduct of purine biotransformation [15,16].

The elevated levels of creatinine, urea, and uric acid brought on by CIS are effectively corrected by the MGLE pretreatment. The pathophysiology of CIS and other cytotoxic drug-induced nephrotoxicity has been proposed to include oxidative stress damage and generation of reactive oxygen species (ROS). The disturbance of the dynamic balance between pro-oxidants and free radicals, which antioxidants scavenge, results in cellular damage [17]. The current investigation shows that CIS administration significantly lowers GSH levels. The poisonous molecule acrolein is the cause of the decrease in GSH content in renal tissues following CIS treatment. Acrolein causes the kidney's tubular cells to necrotise by binding GSH in the plasma membrane, interfering with the antioxidant defence mechanism, and raising ROS. Following CIS administration, a significant decrease in SOD and catalase levels was noted in the current study.

The essential antioxidant enzymes SOD and catalase transform molecules of oxygen into non-toxic byproducts. When ROS levels rise, SOD levels fall [18]. The inhibition of catalase function is the cause of the increase in H2O2 levels. Consequently, the SOD activity is likewise inhibited by catalase deficiency. Increased ROS and lipid peroxidation are the primary causes of the decrease in these antioxidant enzymes. Following CIS therapy, the MGLE pretreatment dramatically raises the levels of GSH, SOD, and catalase [18]. Histopathological analysis and the results of serum markers and antioxidant parameters were connected. Bowman's capsule shrinkage, congestion, blood vessel dilatation, inflammation, and infiltration are all symptoms of CIS treatment. By examining positive effects such no alteration in Bowman's capsule size, mild inflammation, and no blood capillary congestion, the MGLE pretreatment effectively increased the toxic consequences brought on by the CIS renal system [18].

Numerous research in the field of neuroprotection have shown that oxidative stress, LPO, and mitochondrial dysfunction are all involved in the neurotoxicity caused by CIS. CIS causes cytotoxicity by producing reactive oxygen species (ROS). In brain tissues, injection of CIS resulted in reduction in activity of antioxidant defense enzymes, increase in levels of LPO, NO and decrease in concentrations of non-enzymatic components of GSH that prevent/defend against LPO. It is acknowledged that both are associated with oxidative stress and lead to andiscrepancy between the antioxidant capacity of the body and the production of radicals obtained from oxygen [19-22].

The study's findings demonstrate that, in comparison to the normal group, the CIS-treated animals' neural SOD and GSH levels dramatically dropped. The reduction of brain antioxidants

is prevented by simultaneously administering MGLE (400 mg/kg, orally) and CIS therapy. The loss of both zinc and copper, which are necessary for enzyme function, may be the cause of the drop in SOD activity following CIS injection. The superoxide anion generated during the regular metabolic process cannot be scavenged by the reduced SOD activity. LPO can be initiated and progressed by the superoxide anion. Following CIS injection, there is also a reduction in GSH activity. As a result, the brain's capacity to scavenge harmful H2O2 and lipid peroxides was diminished [23-25].

The fact that MGLE restored neuronal SOD and GSH activity indicates that the extract can shield the enzymes. The toxicity of CIS can be significantly increased by GSH deficiency. The neurotoxicity caused by CIS is known to be significantly influenced by free radicals and MGLE clearly shows that the elevated GSH levels provide protection. Oxidative stress is brought on in the brain by ROS and free radicals. CIS becomes more harmful when GSH is depleted. GSH depletion, which seems to be the primary mechanism for LPO and reduced antioxidant enzyme activity, is another cause of LPO. These findings provide credence to the idea that antioxidant system depletion is a contributing factor in the neurotoxicity process observed in rats treated with CIS [23-25]. MGLE's antioxidant action may thus be directly linked to neuroprotection. These results support the theory that antioxidant-rich medicinal herbs may have neuroprotective properties.

CONCLUSION

The current study showed that MGLE (200 mg/kg and 400 mg/kg orally) exhibited hematoprotective, nephroprotective, and neuroprotective properties against cisplatin. Cisplatin caused experimental rats to become nephrotoxic, neurotoxic, and hematotoxic. The herb's possible antioxidant properties, free radical-fighting activity, regulation of serum indicators and safeguarding of histopathological characteristics may all contribute to its effectiveness as a preventative therapy. To prove the reality clinically, more study is needed.

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Iron Homeostasis and Insulin Resistance in Type 2 Diabetes: A Cross-Sectional Study

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ABSTRACT:

Type 2 diabetes mellitus (T2DM) is closely associated with iron biology and oxidative stress. Indian data are, however, sparse regarding the association of abnormal iron indices, particularly hyperferritinemia, with insulin resistance and cardiometabolic risk. Iron status, glycaemia, dyslipidaemia, and oxidative stress in adults with T2DM receiving routine care were investigated in this thesis. (1) compared markers of iron homeostasis (ferritin, transferrin and total iron binding capacity [TIBC]) in adults with T2DM versus age-matched healthy controls; (2) examined the relationship of ferritin with insulin resistance (HOMA IR score) among subjects with well-controlled glycaemia and finally (3) profiled glycaemic indicators, apolipoproteins, inflammatory markers and oxidative stress biomarkers by disease status. A comparative cross-sectional study recruited 100 adults with T2DM and 100 age and sex matched controls (35–65 years). Anthropometry, blood pressure, fasting/post prandial glucose, HbA1c, lipid profile, ApoB/ApoA1, insulin (with HOMA IR), complete blood count, CRP, ESR, vitamins C and E, oxidative stress (MDA, SOD, GST), and iron indices (serum iron, ferritin, transferrin, TIBC) were obtained using standard biochemical and immunoturbidimetric methods; statistics used SPSS 20 with t tests and Pearson correlations (α=0.05).

Compared with controls, the T2DM group had higher BMI (24.83 ± 1.99 vs 23.54 ± 2.54 kg/m²; $p<0.01$) and blood pressure (SBP 134.05 ± 14.25 vs 123.69 ± 8.4 mmHg; DBP 83.12 ± 4.16 vs 80.1 ± 1 mmHg; both $p<0.01$). Fasting glucose (167.3 ± 51.56 vs 85.05 ± 8.54 mg/dL) and PPBS (216.06 ± 69.13 vs 114.15 ± 12.31 mg/dL) were higher (both $p<0.01$). Insulin (15.0 ± 10.21 vs 8.72 ± 11.29 µIU/mL) and HOMA IR (6.11 ± 4.70 vs 1.84 ± 2.40) were elevated ($p<0.01$). Ferritin was markedly higher (155.41 ± 31.91 vs 39.12 ± 21.45 ng/mL; $p<0.01$), while transferrin (244.31 ± 40.35 vs 280.94 ± 60.71 mg/dL; $p<0.01$) and TIBC (251.38 ± 67.72 vs 313.97 ± 64.79 µg/dL; $p<0.01$) were lower. Oxidative stress was higher (MDA 25.64 ± 12.81 vs 5.44 ± 6.04 nmol/mL; $p<0.01$) with lower SOD and GST and reduced vitamins C and E (all $p<0.01$). Ferritin correlated with HOMA IR ($r=0.424$; 95% CI 0.304–0.532; $p<0.001$) among those with satisfactory glycaemic control. Adults with T2DM showed a distinct iron inflammation–oxidative stress phenotype: higher ferritin (with lower transferrin/TIBC), stronger insulin resistance, and heightened oxidative stress. Ferritin's positive association with HOMA IR supports a role for iron dysregulation in insulin resistance in this Indian cohort.

KEY WORDS: Type 2 Diabetes Mellitus; Insulin Resistance; Serum Ferritin; Transferrin; TIBC; Oxidative Stress; Malondialdehyde; Apolipoprotein B.

INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) is increasing around the world, including India, with significant microvascular and macrovascular complications [1]. Indian patients generally exhibit a clustering of dyslipidaemia, hypertension, and inflammation, which enhances vascular risk [2]. In addition to glucose, iron has been identified as an essential mediator of metabolic risk [3]. Elevated ferritin has been linked to insulin resistance, β-cell stress, and unfavourable cardiometabolic phenotypes: in particular, the pathogenetic role of excessive labile iron-mediated production of reactive oxygen species (ROS) and protein glycation mechanisms that lead to impaired activity of the insulin receptor [4]. Data from cohort and mechanistic studies indicate that ferritin and transferrin kinetics (including reduction of Fe^{3+} binding to transferrin by glycation3) are impacted in diabetes, which can further create a vicious circle of oxidative stress and inflammation [5].

Oxidative stress is integral to diabetic vascular pathology: lipid peroxidation by products such as malondialdehyde (MDA) are elevated, while enzymatic antioxidants (e.g., SOD, GST) and antioxidant vitamins (C, E) may be depleted [6]. Dyslipidaemia (notably ApoB-rich lipoproteins) further compounds risk. Despite this, Indian data simultaneously profiling iron indices, insulin resistance, oxidative stress, and emerging lipid markers in a single study remain scarce [7].

Knowledge gap and rationale: While prior work has linked iron status to glucose dysregulation and cardiometabolic risk, there is limited integrative evidence from Indian outpatient populations receiving routine care—especially examining correlations between ferritin and insulin resistance under satisfactory glycaemic control. This study addresses that gap.

Objectives: We sought to (i) compare iron homeostasis markers between T2DM cases and matched controls; (ii) quantify the relationship between ferritin and HOMA IR; and (iii) characterise oxidative stress, antioxidant status, inflammatory markers, and apolipoproteins in the same cohort.

MATERIAL AND METHODS

Study design and setting: This was a comparative cross-sectional study conducted at a diabetic clinic, Kozhikode, Kerala, India, from December 2020 to May 2022.

Participants. Adults aged 35–65 years: 100 with previously diagnosed T2DM on treatment and 100 apparently healthy controls, matched by age and sex. Inclusion required willingness to participate; individuals with life-threatening comorbidities were excluded.

Data collection and assays: Anthropometry and blood pressure were recorded; fasting/post-prandial blood glucose (oxidase–peroxidase method) and lipid profile were measured. ApoB/ApoA1 were quantified by immunoturbidimetry. Oxidative stress was assessed via MDA (thiobarbituric acid reactive substances method) and antioxidant enzymes (SOD, GST); vitamins C and E were measured with standard spectrophotometric protocols. Insulin and HOMA IR were obtained; complete blood count, CRP, ESR, iron, ferritin, transferrin, and TIBC were analysed using standard laboratory methods.

Outcomes: Primary: between-group differences in ferritin, transferrin, and TIBC; association between ferritin and HOMA IR under satisfactory glycaemic control. Secondary: between-group differences in glycaemic, lipid, inflammatory, haematological, and oxidative stress indices.

Table 1. Baseline characteristics of participants (Control vs Test)

Parameter	Control (n=100)	Test (n=100)	t test	p value
Age (years)	47.5 ± 10.56	51.5 ± 10.32	−2.71	<0.01**
Height (cm)	161.94 ± 6.46	162.06 ± 7.58	−0.12	0.904
Weight (kg)	61.96 ± 7.82	64.55 ± 8.07	−2.30	<0.05*
BMI (kg/m²)	23.54 ± 2.54	24.83 ± 1.99	−4.01	<0.01**

Statistical analysis: Data are mean ± SD. Group comparisons used independent samples t tests; Pearson correlations examined relationships among ferritin, insulin, and HOMA IR. Significance was set at $p \leq 0.05$ (SPSS v20).

RESULTS

Participant characteristics: The T2DM group had higher BMI (24.83 ± 1.99 vs 23.54 ± 2.54 kg/m²; p<0.01) and blood pressure (SBP 134.05 ± 14.25 vs 123.69 ± 8.4 mmHg; DBP 83.12 ± 4.16 vs 80.1 ± 1 mmHg; both p<0.01).

Glycaemia and insulin resistance: FBS and PPBS were higher in T2DM (FBS 167.3 ± 51.56 vs 85.05 ± 8.54 mg/dL; PPBS 216.06 ± 69.13 vs 114.15 ± 12.31 mg/dL; both p<0.01). Insulin (15.0 ± 10.21 vs 8.72 ± 11.29 µIU/mL; p<0.01) and HOMA IR (6.11 ± 4.70 vs 1.84 ± 2.40; p<0.01) were elevated.

Lipid and apolipoprotein profile: Traditional lipids were higher in T2DM; ApoB was significantly higher (1.55 ± 0.19 vs 1.46 ± 0.17 g/L; p<0.01), while ApoA1 was modestly higher but not statistically significant (1.29 ± 0.33 vs 1.23 ± 0.13 g/L; p = 0.077).

Oxidative stress and vitamins: MDA was markedly higher (25.64 ± 12.81 vs 5.44 ± 6.04 nmol/mL; p<0.01). Antioxidant enzymes SOD (55.15 ± 37.90 vs 498.73 ± 671.12 U/L; p<0.01) and GST (21.31 ± 18.13 vs 74.89 ± 32.15 ng/mL; p<0.01) were lower. Vitamins C and E were significantly reduced (both p<0.01).

Table 2. Glycaemic parameters in Control vs Test

Parameter	Control (n=100)	Test (n=100)	t test	p value
FBS (mg/dL)	85.05 ± 8.54	167.30 ± 51.56	−15.74	<0.01**
PPBS (mg/dL)	114.15 ± 12.31	216.06 ± 69.13	−14.51	<0.01**
HbA1c (%)	4.87 ± 0.38	5.82 ± 1.16	−7.75	<0.01**

Table 3. Lipid profile in Control vs Test

Parameter	Control (n=100)	Test (n=100)	t test	p value
Total Cholesterol (mg/dL)	178.39 ± 13.57	231.60 ± 27.88	−17.16	<0.01**
HDL C (mg/dL)	47.22 ± 4.48	36.50 ± 3.03	19.80	<0.01**
LDL C (mg/dL)	111.92 ± 10.20	157.96 ± 26.70	−16.11	<0.01**
VLDL C (mg/dL)	20.75 ± 5.06	33.96 ± 11.15	−10.79	<0.01**
Triglycerides (mg/dL)	122.93 ± 18.84	177.46 ± 30.85	−15.08	<0.01**

Inflammation and haematology: CRP and ESR were higher in T2DM; total WBCs, neutrophils, lymphocytes, platelets, RBCs, haematocrit, and MCV were elevated, while MCH/MCHC/RDW indices showed no significant rise. Hb did not differ significantly.

Table 4. Oxidative stress and antioxidant markers

Parameter	Control (n=100)	Test (n=100)	t test	p value
MDA (nmol/mL)	5.44 ± 6.04	25.64 ± 12.81	−14.26	<0.01**
SOD (U/L)	498.73 ± 671.12	55.15 ± 37.90	6.60	<0.01**
GST (ng/mL)	74.89 ± 32.15	21.31 ± 18.13	14.52	<0.01**
Vitamin C (ng/mL)	93.08 ± 40.11	11.07 ± 7.71	20.08	<0.01**
Vitamin E (nmol/mL)	64.94 ± 21.45	7.80 ± 5.42	25.83	<0.01**

Iron homeostasis: Ferritin was substantially higher in T2DM (155.41 ± 31.91 vs 39.12 ± 21.45 ng/mL; p<0.01), whereas transferrin (244.31 ± 40.35 vs 280.94 ± 60.71 mg/dL; p<0.01) and TIBC (251.38 ± 67.72 vs 313.97 ± 64.79 µg/dL; p<0.01) were lower. Among participants with satisfactory glycaemic control, ferritin correlated with HOMA IR (r=0.424; p<0.001) and with insulin (r=0.214; p=0.002). Insulin correlated strongly with HOMA IR (r=0.848; p<0.001).

DISCUSSION

In this clinic-based Indian cohort, T2DM was characterised by higher adiposity and blood pressure; marked hyperglycaemia with elevated insulin/HOMA IR; an atherogenic lipid pattern with higher ApoB; pronounced oxidative stress (high MDA; low SOD/GST and vitamins C/E); systemic inflammation (CRP/ESR); and a distinct iron signature—high ferritin with lower transferrin/TIBC. Significantly, ferritin correlated positively with HOMA IR under satisfactory glycaemic control, strengthening the link between iron stores and insulin resistance. Our results are consistent with previous evidence that has associated ferritin with insulin resistance and T2DM risk [8]. Harrison et al. emphasized the involvement of iron in glucose toxicity and insulin action [9]. A meta-analysis by Yang et al. supported the relationship between abnormal iron homeostasis and T2DM [10].

Conway et al. In population studies, body iron was independently associated with serum insulin and glucose [11]. While mechanistically hepcidin is known to modulate iron flux, transferrin glycation may reduce the binding capacity of iron and increase labile iron, leading to ROS and inflammation – consistent with our high ferritin and oxidative stress [12].

The documented lower transferrin/TIBC in T2DM likely mirrors inflammation-induced negative acute phase-reactants and glycation-mediated function impairment of transferrin, as evidenced by in vitro and clinical studies. In concert, they may attenuate iron binding and exaggerate oxidative damage in line with our increased MDA and depleted antioxidant levels. Our pattern of oxidative stress—increased MDA, decreased SOD/GST, and lower vitamins C/E—is consistent with traditional diabetes reports and newer endothelial/metabolic dysregulation studies that emphasize ROS damage.

On lipids, ApoB no question complements the idea that higher ApoB is better than LDL C as a risk marker, significant in DM, where dense LDL rules the roost. Discrepancies with some literature (for example, the lack of a substantial increase in ApoA1 in our study) may result from the status of treatment, diet, or background inflammation in our cohort.

Strengths are age/sex matching, a large biomarker panel (glycaemic, lipid, inflammatory-oxidative-iron indices), and formal correlation of ferritin against insulin resistance over an interval clinically pertinent. The thesis addressed these limitations: cross-sectional nature (i.e., no causality), single-centre recruitment, incomplete formal risk assessment for diabetic complications, and potential residual confounding (dietary iron; hepcidin status; medication effects). Limitations Analytic choices (e.g., use of HOMA IR rather than clamp studies; lack of measurement of hepcidin levels) limit mechanistic inference.

CONCLUSION

This study demonstrates that, in Indian adults with T2DM, iron dysregulation (high ferritin with lower transferrin/TIBC) coexists with insulin resistance and heightened oxidative stress. Ferritin relates positively to HOMA IR under satisfactory glycaemic control. These data support integrating iron indices into cardiometabolic risk appraisal and motivate prospective/interventional work (e.g., defining optimal ferritin targets, clarifying hepcidin dynamics) in Indian settings.

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Conflict of interest: The authors declare no competing interests.

Data availability: The identified data underlying this study's findings (biomarker matrices and statistical code) are available from the corresponding author upon reasonable request, subject to ethical approvals and institutional policies.

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Knowledge, Attitude, and Practice of Digital Dentures Among Practising Dentists in the Mumbai Metropolitan Region: A Cross-Sectional Questionnaire Study

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ABSTRACT:

Digital dentures are increasingly being introduced into prosthodontic practice because of their potential to improve workflow standardization, denture accuracy, and record preservation. However, their successful clinical adoption depends on the knowledge, attitude, and practical exposure of dentists. To assess the knowledge, attitude, and practice regarding digital dentures among practising dentists in the Mumbai Metropolitan Region. A questionnaire-based observational cross-sectional study was conducted among practising dentists in the Mumbai Metropolitan Region using a web-based survey. A structured close-ended questionnaire assessed demographic details and responses related to knowledge, attitude, practice, perceived barriers, and future prospects of digital dentures. Data were analyzed using descriptive statistics, and qualification-wise comparisons were performed using tests for categorical variables. A p value of less than 0.05 was considered statistically significant. A total of 111 responses were analyzed. Most respondents had heard of digital dentures (93.7%), and 93.7% considered them a valuable innovation in prosthodontics. However, only 24.3% had tried fabricating digital dentures in clinical practice, and 24.3% reported hands-on experience. Formal curricular learning was reported by 30.6% of respondents. Most participants felt that affordability depended on locality (68.5%), and 50.5% reported multiple concurrent barriers to learning or practice. MDS respondents showed significantly greater clinical exposure, curricular learning, and academic exposure than BDS respondents. Practising dentists showed high awareness and a favorable attitude toward digital dentures, but practical exposure and routine use remained limited. The findings indicate a gap between awareness and implementation and support the need for stronger educational and hands-on training opportunities.

KEY WORDS: DDigital dentures; CAD-CAM dentures; Digital dentistry; Prosthodontics; Questionnaire surveyQuestionnaire survey

INTRODUCTION

Edentulism remains an important prosthodontic problem, particularly in older adults, and complete dentures continue to play a major role in restoring mastication, speech, esthetics, and quality of life. Conventional complete denture fabrication has been used successfully for many years, but it involves multiple clinical and laboratory steps and is often time-consuming and technique-sensitive. In recent years, digital workflows have gained increasing attention because they offer a more standardized approach to denture fabrication and may reduce chairside and laboratory time while preserving digital records for future remakes ([1,2]

Digital complete dentures are fabricated using computer-aided design and computer-aided manufacturing technologies, usually through subtractive milling or additive three-dimensional printing. These methods have expanded the scope of removable prosthodontics by introducing new options for denture design, manufacturing precision, and workflow simplification. Reviews of currently available CAD-CAM complete denture systems have noted that digital workflows are becoming more clinically relevant as materials, software, and manufacturing systems continue to improve [3].

Accuracy of the denture base is one of the most important factors influencing retention, stability, and adaptation of complete dentures. Experimental studies have shown that digitally fabricated denture bases can achieve clinically acceptable adaptation. [4] reported that both milled and digital light processing-generated mandibular denture bases demonstrated intaglio surface adaptation within a clinically acceptable range. Similarly, [5] found that digitally fabricated maxillary denture bases showed favorable trueness and tissue surface adaptation when compared with conventional pack-and-press methods [6]. further observed that CAD-CAM milled and rapid prototyping methods showed better overall accuracy than conventional injection molding in their comparative evaluation of denture base fabrication techniques [4,5 & 6].

Despite these advancements, successful incorporation of digital dentures into routine practice depends not only on the technology itself but also on the clinician's knowledge, acceptance, and practical exposure. In a metropolitan region such as Mumbai, where digital dentistry is gradually becoming more visible in academic and private practice settings, it is important to understand how practising dentists perceive and use digital denture technology. Therefore, the present study was undertaken to assess the knowledge, attitude, and practice of digital dentures among practising dentists in the Mumbai Metropolitan Region.

MATERIALS AND METHODS

Study design and setting: This questionnaire-based observational cross-sectional study was conducted to assess the knowledge, attitude, and practice regarding digital dentures among practising dentists in the Mumbai Metropolitan Region (MMR). The study was carried out as a web-based survey, which allowed inclusion of respondents from different clinical and academic practice settings within the region. The study was conducted according to the approved study protocol and in accordance with accepted ethical principles for questionnaire-based research. The respondents were informed about the objectives of the study before participation, and confidentiality of their responses was maintained throughout the study. As the study was non-interventional and based solely on an anonymous web-based questionnaire, no clinical procedure was involved.

Study population and eligibility criteria: The study population comprised practising dentists working in the Mumbai Metropolitan Region. Both Bachelor of Dental Surgery (BDS) graduates and Master of Dental Surgery (MDS) practitioners were considered eligible for participation. Dentists practising outside the Mumbai Metropolitan Region and undergraduate dental students were excluded from the study. Since the study was based on voluntary participation in an online questionnaire, withdrawal criteria were not applicable.

Sample size and survey approach: A target sample of 200 practising dentists was planned for the survey. The questionnaire was circulated electronically among eligible participants, and responses received from those who met the inclusion criteria were considered for analysis. Only completed responses were included in the final dataset. The web-based approach was chosen to improve accessibility and facilitate participation from dentists across different areas of the metropolitan region.

Study instrument: Data were collected using a structured, close-ended questionnaire designed for this study. The questionnaire included items related to demographic details such as qualification and specialty, followed by questions assessing knowledge, attitude, and practice regarding digital dentures. The knowledge component assessed awareness of the term digital dentures, prior exposure to the subject in the academic curriculum, and understanding of the digital denture workflow. The attitude component included questions related to the perceived value of digital dentures, their future role in prosthodontic practice, affordability, the need for additional undergraduate training, and confidence in using the technology in future. The practice component assessed previous fabrication of digital dentures, hands-on experience, and extent of academic exposure. Additional questions were included to identify perceived barriers to learning or practising digital denture fabrication and the expected impact of digital dentures on patient care.

Data collection procedure: The questionnaire was distributed through online platforms to practising dentists in the Mumbai Metropolitan Region. Before participation, the respondents were informed about the purpose and nature of the study. They were assured that the information provided by them would be kept confidential and used only for academic and research purposes. No open-ended questions were included in the survey.

Study variables and outcome measures: The primary outcome of the study was the assessment of knowledge, attitude, and practice related to digital dentures among practising dentists in the Mumbai Metropolitan Region. The main study variables included awareness of digital dentures, curricular learning, perception of their value in prosthodontics, opinion regarding their future use, confidence in adopting the

technology, actual clinical exposure, and hands-on experience. Secondary observations included perceived barriers to learning or implementation, opinion regarding affordability, expected impact on patient care, and views on the integration of digital denture training into the undergraduate curriculum.

Data management and statistical analysis: The collected responses were compiled in a master chart and reviewed for completeness before analysis. Categorical variables were summarized using frequencies and percentages. The findings were presented in tabular form for clarity. For subgroup comparisons, qualification-based differences between BDS and MDS respondents were assessed using appropriate tests for categorical data, such as the chi-square test or Fisher's exact test wherever required. A p value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A total of 111 completed questionnaire responses were included in the analysis. Of these, 69 respondents (62.2%) were MDS practitioners and 42 (37.8%) were BDS practitioners. With respect to specialty distribution, 34 respondents (30.6%) belonged to Prosthodontics, Crown and Bridge, followed by Pediatric and Preventive Dentistry and Periodontology with 14 respondents (12.6%) each. Conservative Dentistry and Endodontics and Oral Medicine and Radiology each contributed 7 respondents (6.3%). In 35 responses (31.5%), the branch was not applicable or not specified, corresponding largely to BDS participants. The distribution of participant characteristics is shown in Table 1.

Table 1. Participant characteristics

Characteristic	Category	n (%)
Qualification	BDS	42 (37.8)
	MDS	69 (62.2)
Specialty/branch	Not applicable/not specified	35 (31.5)
	Prosthodontics, Crown & Bridge	34 (30.6)
	Pediatric and Preventive Dentistry	14 (12.6)
	Periodontology	14 (12.6)
	Conservative Dentistry and Endodontics	7 (6.3)
	Oral Medicine and Radiology	7 (6.3)

Knowledge and practice related to digital dentures: Awareness regarding digital dentures was high, with 104 respondents (93.7%) reporting that they had heard of the term. However, only 27 respondents (24.3%) had tried to fabricate a digital denture in their clinic, and an identical proportion reported hands-on experience with digital denture fabrication. Learning about digital dentures through the formal curriculum was reported by 34 respondents (30.6%), while 77 (69.4%) stated that they had not learned about the topic in their curriculum. Regarding academic exposure, the most common response was "rarely" in 56 respondents (50.5%), followed by "never" in 21 (18.9%), "frequently" in 20 (18.0%), and "occasionally" in 14 (12.6%). These findings are presented in Table 2.

Table 2. Knowledge and practice related to digital dentures

Variable	Response	n (%)
Heard of the term digital dentures	Yes	104 (93.7)
	No	7 (6.3)
Tried to fabricate digital dentures in clinic	Yes	27 (24.3)
	No	84 (75.7)
Learned about digital dentures in curriculum	Yes	34 (30.6)
	No	77 (69.4)
Hands-on experience with digital denture fabrication	Yes	27 (24.3)
	No	84 (75.7)
Exposure to digital denture content in academic curriculum	Frequently	20 (18.0)
	Occasionally	14 (12.6)
	Rarely	56 (50.5)
	Never	21 (18.9)

Attitude, perceived barriers, and future prospects: Most respondents expressed a favorable attitude toward digital dentures. A total of 62 respondents (55.9%) strongly agreed and 42 (37.8%) agreed that digital dentures are a valuable innovation in prosthodontics. Regarding future replacement of conventional dentures, 63 respondents (56.8%) believed that digital dentures would completely replace conventional dentures, whereas 48 (43.2%) felt that a combination of both approaches would be ideal. With respect to affordability, 76 respondents (68.5%) felt that affordability depended on locality, 28 (25.2%) considered digital dentures affordable, and 7 (6.3%) considered them non-affordable.

For undergraduate training, 62 respondents (55.9%) strongly agreed and 28 (25.2%) agreed that students should receive more training on digital denture fabrication. Confidence in using digital denture technology in the future was reported as very confident by 48 respondents (43.2%), somewhat confident by 28 (25.2%), and requiring further learning and training by another 28 (25.2%), while 7 respondents (6.3%) reported that they were not confident. The most common perceived barrier was the presence of multiple simultaneous barriers, reported by 56 respondents (50.5%). With regard to future prospects, 76 respondents (68.5%) expected a revolutionary improvement in patient care, and 55 (49.5%) strongly agreed while 35 (31.5%) agreed that digital denture training should be integrated more strongly into the undergraduate curriculum. These data are summarized in Table 3.

Table 3. Attitude toward digital dentures, perceived barriers, and future prospects

Variable	Response	n (%)
Digital dentures are a valuable innovation in prosthodontics	Strongly agree	62 (55.9)
	Agree	42 (37.8)
	Neutral	7 (6.3)
Belief that digital dentures will replace conventional dentures in future	Yes, completely	63 (56.8)
	A combination of both is ideal	48 (43.2)
Affordability of digital dentures for patients	Affordable	28 (25.2)
	Depends on locality	76 (68.5)
	Non-affordable	7 (6.3)
Need for more undergraduate training	Strongly agree	62 (55.9)
	Agree	28 (25.2)
	Neutral	14 (12.6)
	Disagree	7 (6.3)
Confidence in ability to understand and use digital denture technology in future	Very confident	48 (43.2)
	Somewhat confident	28 (25.2)
	Need some learning and training sessions	28 (25.2)
	Not confident	7 (6.3)
Perceived barriers to learning or practicing digital denture fabrication	All above	56 (50.5)
	Limited resources	14 (12.6)
	Lack of teaching in curriculum	14 (12.6)
	Lack of hands-on training	14 (12.6)
	High cost of equipment	7 (6.3)
	None	6 (5.4)
Perceived impact on patient care	Revolutionary improvement	76 (68.5)
	Moderate improvement	35 (31.5)
Recommendation for greater integration into undergraduate curriculum	Strongly agree	55 (49.5)
	Agree	35 (31.5)
	Neutral	7 (6.3)
	Disagree	14 (12.6)

Comparison according to qualification: Qualification-wise comparison demonstrated statistically significant differences for several variables. Previous clinical fabrication of digital dentures was reported by 27 of 69 MDS respondents (39.1%), whereas none of the 42 BDS respondents reported such experience ($p<0.001$). Similarly, learning about digital dentures in the curriculum was more frequent among MDS respondents than BDS respondents (39.1% vs 16.7%; $p=0.023$). A significant difference was also observed in perception of digital dentures as a valuable innovation: 48 MDS respondents (69.6%) strongly agreed compared with 14 BDS respondents (33.3%), while neutrality was reported only among BDS respondents (16.7%) ($p<0.001$).

Exposure to digital denture-related content also differed significantly between the two qualification groups ($p<0.001$). Frequent exposure was reported only among MDS respondents (29.0%), whereas "never" was more common among BDS respondents (33.3% vs 10.1%). Confidence regarding future use showed a significant association with qualification ($p=0.034$), with "very confident" being reported by 50.0% of BDS respondents and 39.1% of MDS respondents, while lack of confidence was reported only among MDS respondents (10.1%). Affordability perception also differed significantly between groups ($p=0.015$). In contrast, no statistically significant association with qualification was observed for awareness of the term digital dentures ($p=0.084$), belief regarding complete future replacement of conventional dentures ($p=0.148$), perceived impact on patient care ($p=0.914$), or recommendation for curricular integration ($p=0.157$). The detailed comparison is presented in Table 4.

The present study showed that awareness of digital dentures among practising dentists in the Mumbai Metropolitan Region was high, but this did not translate into equivalent levels of clinical use or hands-on experience. This gap between

awareness and implementation is clinically important because adoption of digital dentures depends not only on knowing the term, but also on exposure to scanning, digital design, manufacturing workflow, and post-processing steps. A similar pattern was reported by [7], who found high awareness of digital dentures among dentists, while actual practice and confidence remained lower. Likewise, [8] reported that perception toward digital dentistry was generally favorable, but emphasized that stronger educational exposure and practical training were still needed for meaningful clinical integration.

Table 4. Qualification-wise comparison of selected variables

Variable	Response	BDS n (%)	MDS n (%)	p value
Heard of the term digital dentures	Yes	42 (100.0)	62 (89.9)	0.084
	No	0 (0.0)	7 (10.1)	
Tried to fabricate digital dentures in clinic	Yes	0 (0.0)	27 (39.1)	<0.001
	No	42 (100.0)	42 (60.9)	
Learned about digital dentures in curriculum	Yes	7 (16.7)	27 (39.1)	0.023
	No	35 (83.3)	42 (60.9)	
Digital dentures are a valuable innovation in prosthodontics	Strongly agree	14 (33.3)	48 (69.6)	<0.001
	Agree	21 (50.0)	21 (30.4)	
	Neutral	7 (16.7)	0 (0.0)	
Affordability of digital dentures for patients	Affordable	7 (16.7)	21 (30.4)	0.015
	Depends on locality	35 (83.3)	41 (59.4)	
	Non-affordable	0 (0.0)	7 (10.1)	
Confidence in ability to understand and use digital denture technology in future	Very confident	21 (50.0)	27 (39.1)	0.034
	Somewhat confident	7 (16.7)	21 (30.4)	
	Need some learning and training sessions	14 (33.3)	14 (20.3)	
	Not confident	0 (0.0)	7 (10.1)	
Exposure to digital denture content in academic curriculum	Frequently	0 (0.0)	20 (29.0)	<0.001
	Occasionally	7 (16.7)	7 (10.1)	
	Rarely	21 (50.0)	35 (50.7)	
	Never	14 (33.3)	7 (10.1)	
Perceived impact on patient care	Revolutionary improvement	28 (66.7)	48 (69.6)	0.914
	Moderate improvement	14 (33.3)	21 (30.4)	
Recommendation for greater integration into undergraduate curriculum	Strongly agree	21 (50.0)	34 (49.3)	0.157
	Agree	14 (33.3)	21 (30.4)	
	Neutral	0 (0.0)	7 (10.1)	
	Disagree	7 (16.7)	7 (10.1)	

The positive attitude observed in the present study is understandable in light of the growing evidence supporting digital complete denture workflows. Systematic review evidence suggests that CAD-CAM complete dentures are not inferior to conventional dentures and may offer additional advantages such as better retention, improved mechanical properties, reduced chairside time, and preservation of digital records for future remakes [10]. Experimental studies have also shown that CAD-CAM dentures, particularly milled dentures, demonstrate superior fit compared with conventionally fabricated dentures, which may explain why many clinicians increasingly regard digital dentures as a valuable innovation in prosthodontics [11]. Similarly, [12] found that milled PMMA denture bases showed better adaptation than printed and conventionally fabricated denture bases, supporting the perception that digital methods may improve prosthesis accuracy.

In the present study, MDS respondents showed greater curricular exposure, greater clinical experience, and significantly more prior fabrication of digital dentures than BDS respondents. This finding is logical because postgraduate training provides more specialty-based exposure, especially in prosthodontics and related digital workflows. The importance of such training has been highlighted in earlier literature [13]. demonstrated that a complete denture workflow based on intraoral scans is feasible, but such techniques require familiarity with digital records, maxillomandibular relationship registration, and appropriate case selection [14] also noted that although digital workflows may improve clinician-reported

and patient-related outcomes in several aspects, adoption still depends on practical understanding, operator learning curve, and clinical infrastructure. Therefore, the present finding that many respondents supported stronger undergraduate integration of digital denture training appears justified and reflects a real educational need.

Another notable finding was that affordability was considered dependent on locality by most respondents, and the most frequently reported barriers were multiple concurrent obstacles, limited resources, lack of curricular teaching, lack of hands-on training, and high equipment cost. These responses reflect the current practical situation in removable digital prosthodontics. Although digital denture systems may reduce appointments and improve workflow efficiency, the required infrastructure remains expensive and is not uniformly available across all practice settings. Earlier survey-based work has also identified cost, equipment access, and insufficient practical training as major barriers to adoption [7,8]. Clinically, this suggests that digital dentures are presently being viewed more as a promising adjunct or evolving alternative rather than a universal replacement for conventional dentures in all settings.

This study has certain limitations. It was a cross-sectional, questionnaire-based survey and therefore depended on self-reported responses, which may be influenced by recall bias or response bias. The sample was restricted to practising dentists in one metropolitan region, so the findings may not be generalizable to other regions or institutional settings. In addition, the questionnaire assessed perceived knowledge and practice rather than objectively measured competence. Future studies should include multicentric samples, validated scoring systems for knowledge and attitude, and subgroup analysis based on specialty, years of experience, and type of practice. Longitudinal or interventional studies evaluating the effect of structured teaching modules, hands-on workshops, and continuing dental education programs on actual adoption of digital denture workflows would provide stronger evidence for curriculum planning and clinical implementation.

CONCLUSION

Digital dentures were widely recognized by practising dentists in the Mumbai Metropolitan Region and were generally viewed positively as an emerging advancement in prosthodontics. However, actual clinical use, hands-on experience, and curricular exposure remained limited, indicating a gap between awareness and implementation. Within the limits of this study, the findings suggest a need for stronger educational exposure and practical training to support more confident and effective adoption of digital denture technology.

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In vitro Clonal Propagation of Medicinally Valuable sp. *Centella asiatica* (L.) for Conservation and Sustainable Utilization

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ABSTRACT:
In Vitro clonal propagation of medicinally important plant species: Ensuring High Alkaloid Production and Genetic Stability in *Centella asiatica* (L.). This clearly conveys both the purpose conservation and maintaining biochemical traits. The plant is believed to be native to tropical and subtropical regions, particularly Sri Lanka, India, Madagascar, South Africa, and Malaysia. It grows abundantly in moist, swampy, and shaded environments such as riverbanks, paddy fields, and wetlands, where it forms a dense green cover. *Centella asiatica* (L.) belong to the Apiaceae family important valuable alkaloid medicinal herb and It helps in curing many diseases like Memory-Enhancing [7], Anti-ulcer, Antitumor, Antitumor, Cardio-protective, Antiviral Activity etc. because of this plant immediate conservation and develop cost effective micropropagation technique with help of suitable combination and concentration with MS media reduce and minimize contamination percentage. MS basal medium was supplemented with 3% sucrose, seven different concentrations of BAP with 0.5 mg/l NAA, separately. It was noted that MS + 3% sucrose, gelled with 0.8% agar + The best response in the present study was observed at 3.0 mg/L BAP + 3.0 mg/L IAA, which showed maximum initiation (85%), shoot proliferation (95%), and elongation (90%) along with excellent rooting. Bavistin and $HgCl_2$ treatments significantly reduced contamination rates, with maximum effectiveness observed at higher concentrations and optimal exposure times. High contamination rates during initial culture establishment have also been reported in *Centella asiatica*, emphasizing the importance of effective sterilization protocols.

KEY WORDS: *Centella asiatica*, Micropropagation, conservation, Medicinal, MS media, PGR and Contamination rate.

INTRODUCTION

Medicinal plants have been an essential component of human healthcare systems since ancient times, serving as a primary source of therapeutic agents for the treatment of various diseases. Traditional systems such as Ayurveda, Unani, and Traditional Chinese Medicine have extensively relied on plant-based remedies long before the development of modern pharmaceuticals [3]. Even today, a significant proportion of the global population, particularly in developing countries, depends on herbal medicine due to its affordability, accessibility, and relatively fewer side effects.

The medicinal value of plants is attributed to the presence of diverse bioactive compounds, including alkaloids, flavonoids, tannins, and phenolic substances. These phytochemicals exhibit a wide range of pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, and anticancer effects. Their combined action enhances the therapeutic

potential of medicinal plants, making them highly valuable in both traditional and modern healthcare systems [10].

Among the various medicinal plants, *Centella asiatica* (L.), belonging to the family Apiaceae, is one of the most important herbs due to its extensive pharmacological properties. Commonly known as Gotu Kola or Mandukaparni, it is widely distributed in tropical and subtropical regions, including India, Sri Lanka, China, and Africa. The plant thrives in moist and shaded environments and is characterized by its creeping growth habit and kidney-shaped leaves. Traditionally, C. asiatica has been used for enhancing memory and cognitive function, promoting wound healing, reducing inflammation, and managing stress and anxiety.

The therapeutic efficacy of C. asiatica is primarily due to its rich phytochemical composition, particularly triterpenoids such as asiaticoside and madecassoside, along with flavonoids and phenolic compounds. These constituents contribute to its antioxidant, neuroprotective, antimicrobial, and anti-inflammatory activities [9]. Owing to these properties, the plant has gained considerable importance in pharmaceutical, cosmetic, and nutraceutical industries. (South African Journal of Botany 2021).

Despite its immense medicinal value, C. asiatica faces several challenges related to natural propagation. Poor seed viability, slow growth rate, and increasing commercial demand have led to overexploitation of natural populations, resulting in a decline in its availability and genetic diversity. Conventional propagation methods are insufficient to meet the growing demand and ensure sustainable utilization [12].

In this context, plant tissue culture techniques, particularly *in vitro* propagation, offer a promising alternative for the rapid multiplication and conservation of C. asiatica. Micropropagation enables the production of large numbers of disease-free and genetically uniform plantlets under controlled conditions, independent of seasonal variations. Therefore, the present study focuses on developing an efficient *in vitro* propagation protocol for *Centella asiatica* to support its large-scale production, conservation, and sustainable utilization.

The study demonstrates that *in vitro* propagation is an efficient and reliable method for the mass multiplication of *Centella asiatica*. Rapid shoot initiation and successful culture establishment confirmed the suitability of tissue culture techniques for this medicinal plant [2]. Murashige and Skoog (MS) medium proved effective in supporting optimal explant growth. Among plant growth regulators, BAP significantly enhanced multiple shoot induction, while auxins such as NAA and IBA promoted efficient root formation. The combined application of cytokinins and auxins resulted in well-developed plantlets with balanced shoot and root systems. A high survival rate during acclimatization indicated the physiological stability and adaptability of regenerated plants. The findings support previous studies and highlight the potential of this protocol for large-scale production, conservation, and sustainable utilization of *Centella asiatica* in pharmaceutical and cosmetic applications.

MATERIALS AND METHODS

Collection of Plant Material: Healthy plants of *Centella asiatica* were collected from the medicinal nursery established under the Biodiversity Division of the State Forest Research Institute, Jabalpur, Madhya Pradesh, India. The collected plants were maintained under suitable conditions prior to experimentation.

Selection of Explants: Different explants such as nodal segments, root segments, and leaf tissues were considered for *in vitro* culture. Among these, nodal segments were found to be most suitable for shoot induction due to the presence of axillary buds. Leaf explants were mainly used for callus formation, while root explants exhibited a comparatively low response in shoot regeneration. Therefore, nodal and root explants were selected for the present study, with nodal segments showing the highest regeneration efficiency.

Sterilization Procedure: Sterilization is a critical step in plant tissue culture to eliminate microbial contamination. Since the culture medium is nutrient-rich, it favors the growth of microorganisms; hence, strict aseptic conditions were maintained.

Physical Sterilization Methods

Dry Heat Sterilization

- Glassware such as Petri dishes, flasks, and pipettes were sterilized using a hot air oven or microwave at 160–180°C for 2–3 hours.

Moist Heat Sterilization

- Culture media and instruments were sterilized in an autoclave at 121°C and 15 psi pressure for 15–20 minutes.
- **Filtration:**
- Heat-sensitive substances such as plant growth regulators (BAP, NAA) and vitamins were sterilized using membrane filters (0.22 μm).

Chemical Sterilization: Surface sterilization of explants was carried out using the following chemicals:

- Ethanol (70%) for 30–60 seconds for initial disinfection
- Mercuric chloride ($HgCl_2$) at varying concentrations (0.01–0.10%) for 2–5 minutes
- Bavistin (1–4%) as an antifungal treatment for 5–25 minutes

After chemical treatment, explants were thoroughly rinsed with sterile distilled water to remove traces of sterilants.

Culture Media: Murashige and Skoog (MS) medium (1962) was used as the basal medium for *in vitro* culture. It contains

essential macro- and micronutrients, vitamins, and a carbon source necessary for plant growth and development.

Preparation of MS Medium: The MS medium was prepared using standard stock solutions:

- Macronutrients (Stock I, 20×)
- Micronutrients (Stock II, 200×)
- Iron source (Stock III)
- Vitamins (Stock IV, 200×)

To the medium, 3% (30 g/L) sucrose was added as a carbon source. The pH was adjusted to 5.7–5.8 using 1N HCl or 1N NaOH before adding 0.8% agar (8 g/L) for solidification. The medium was then sterilized by autoclaving. Plant growth regulators (PGRs) such as BAP, NAA, and IBA were added as required depending on the experimental objectives.

Surface Sterilization Protocol: Explants were first treated with Bavistin solution (1–4%) for 5–25 minutes to remove fungal contamination. This was followed by treatment with mercuric chloride (0.01–0.10%) for 2–5 minutes under aseptic conditions. Finally, explants were rinsed multiple times with sterile distilled water before inoculation.

Preparation of MS Medium (1 Litre): Murashige and Skoog (MS) medium were prepared following standard procedures. Initially, 500 ml of double distilled water (DDW) was taken in a conical flask, and 30 g of sucrose was added and dissolved completely. Thereafter, 50 ml of Stock I (macronutrients), 5 ml each of Stock II (micronutrients), Stock III (iron source), and Stock IV (vitamins) were added sequentially with continuous stirring [12].

The required concentration of plant growth regulators (PGRs) was then added. The volume was made up to 1000 ml using DDW. The pH of the medium was adjusted to 5.7–5.8 using 1N HCl or 1N NaOH. Subsequently, 8 g/L agar was added as a solidifying agent, and the medium was heated in a microwave to dissolve the agar completely. The prepared medium was poured into culture tubes or bottles and allowed to solidify. Finally, the media were sterilized in an autoclave at 121°C and 15 psi pressure for 30 minutes.

Inoculation Procedure: All inoculation procedures were carried out under aseptic conditions. Sterilized explants were transferred into pre-sterilized culture vessels using sterile forceps. The explants were trimmed using a scalpel to remove damaged portions and then inoculated onto the culture medium under flame or within a laminar airflow cabinet. The culture vessels were sealed properly with caps or closures and further secured with parafilm or tape. Each culture was labeled with the name of the explant and date of inoculation before being transferred to the culture room.

Fresh Culture Establishment: Healthy and phenotypically superior plants were selected as the source of explants. The collected explants were washed thoroughly under running tap water to remove surface contaminants. This was followed by washing with detergent (Extran) to reduce microbial load and rinsing with double distilled water (3–4 times).

Explants were then treated with Bavistin solution to eliminate fungal contamination, followed by repeated washing with DDW. Surface sterilization was carried out using 0.1% mercuric chloride ($HgCl_2$) for 5 minutes under aseptic conditions. After sterilization, explants were rinsed 4–5 times with sterile double distilled water to remove any traces of $HgCl_2$. The sterilized explants were aseptically inoculated onto culture media using sterile forceps and scissors under a laminar airflow chamber. Cultures were maintained in a culture room at 25 ± 2°C.

Sub-culturing: Sub-culturing was performed to maintain and multiply the cultures. It involves transferring explants from an old medium to a fresh nutrient medium to ensure continuous growth and development. Before sub-culturing, the laminar airflow chamber was sterilized using UV light for 20 minutes. Explants were carefully removed from the culture vessels using sterile forceps and transferred to a sterile Petri plate. The ends of the explants were trimmed using a sterile scalpel and then inoculated into fresh media under aseptic conditions. The culture vessels were sealed, labeled, and transferred back to the culture room for further growth.

Culture Room Conditions

The culture room was maintained under controlled environmental conditions to ensure optimal growth of cultures. The temperature was maintained at 25 ± 2°C using air conditioners. A photoperiod of 16 hours light and 8 hours dark was provided, with light intensity ranging between 2000–3000 lux. Relative humidity was maintained at 60–70%. Special racks or shelves made of glass or plywood were used for placing culture vessels. Proper precautions were taken to prevent disturbance and contamination.

Hardening of Plantlets: Regenerated plantlets were transferred to a mist chamber for acclimatization. The process of hardening helps the plantlets to gradually adapt from *in vitro* to ex vitro conditions, ensuring higher survival rates under natural environmental conditions.

Growth Regulators: Different concentrations of plant growth regulators were used to study their effect on shoot initiation, proliferation, elongation, and rooting.

- BAP (6-Benzylaminopurine): 0.0, 1.0, 2.0, 3.0, 4.0 mg/L
- IAA (Indole-3-acetic acid): 0.0, 1.0, 2.0, 3.0, 4.0 mg/L

The combination of BAP and IAA at 3.0 mg/L each showed the best response, with 85% shoot initiation and 95% shoot proliferation along with healthy rooting. Higher concentrations (4.0 mg/L) resulted in excessive callus formation and reduced rooting efficiency. These conditions were found to be optimal

for the *in vitro* growth and development of *Centella asiatica* [14].

RESULTS AND DISCUSSION

The present study was conducted to evaluate the effect of different concentrations of BAP and IAA on the *in vitro* growth and development of *Centella asiatica* at different culture periods (7, 14, 21 and 28 days).The results clearly indicated that growth response varied significantly with different concentrations of plant growth regulators. Explants cultured on hormone-free medium (0.0 mg/L BAP + 0.0 mg/L IAA) showed no response, confirming that exogenous supply of plant growth regulators is essential for *in vitro* morphogenesis. Similar observations have been reported in *Centella asiatica*, where growth regulator-free media failed to induce significant shoot or root development. [10].

Among different explants types, nodal explants showed the highest response, followed by leaf and root explants. This is consistent with earlier findings that nodal segments possess pre-existing meristems, making them more responsive for shoot induction in tissue culture.

With the application of growth regulators, a gradual improvement in growth response was observed. The treatment containing 1.0 mg/L BAP + 1.0 mg/L IAA showed moderate response, with 45% initiation, 55% shoot proliferation and 60% elongation, but poor rooting. Similar moderate responses at lower hormone concentrations have been reported, indicating that suboptimal levels of cytokinin and auxin limit morphogenetic efficiency [15].

The best response in the present study was observed at 3.0 mg/L BAP + 3.0 mg/L IAA, which showed maximum initiation (85%), shoot proliferation (95%), and elongation (90%) along with excellent rooting. These findings are in agreement with previous studies reporting that balanced combinations of cytokinins (BAP/BA) and auxins (IAA/NAA) significantly enhance shoot multiplication and plant regeneration in *Centella asiatica* [8].

A slightly lower but still effective response was observed at 2.0 mg/L BAP + 2.0 mg/L IAA, indicating that optimal hormonal balance is critical for coordinated shoot and root development. The synergistic interaction between cytokinin and auxin has been widely reported to regulate organogenesis, where cytokinin promotes shoot induction and auxin supports root formation. [15].

However, further increase in hormone concentration (4.0 mg/L BAP + 4.0 mg/L IAA) resulted in reduced growth performance, including decreased elongation and callus formation [3]. This observation supports earlier reports that excessive concentrations of growth regulators can disrupt endogenous hormonal balance and lead to abnormal growth or callogenesis instead of organized plant development.

Surface sterilization treatments also played a crucial role in successful culture establishment. Bavistin and $HgCl_2$ treatments significantly reduced contamination rates, with maximum effectiveness observed at higher concentrations and optimal exposure times. High contamination rates during initial culture establishment have also been reported in *Centella asiatica*, emphasizing the importance of effective sterilization protocols.

Table 1. Optimization of bavistin concentration and exposure duration for effective surface sterilization of Centella asiatica explants

Experiment No	Concentration of Bavistin in %	Time Duration (min)	Contamination rate in (%)
T 0	Control	-	90%
T 1	0.2	2min	70%
T 2	0.4	4min	50%
T 3	0.6	5min	30%
T 4	1.0	10min	3%
T 5	0.8	15min	20%

Table 2. Effect of varying HgCl2 concentrations on microbial contamination control in Centella asiatica tissue culture

Experiment No	Concentration of Hgcl2	Time Duration (min)	Contamination rate in (%)
T 0	Control	-	90%
T 1	0.2	2min	70%
T 2	0.4	3min	50%
T 3	0.6	4min	30%
T 4	1.0	5min	2%
T 5	0.8	6min	10%

Table 3. Comparative regeneration potential of Nodal, Leaf and Root explants of Centella asiatica under In Vitro conditions

S.No	Explants type	Response Observation
1.	Nodal explants	High response
2.	Leaf explants	Moderate response
3.	Root explants	Low response

The use of Murashige and Skoog (MS) medium provided essential nutrients for optimal growth and development of explants. Previous studies have also confirmed that MS

medium supplemented with appropriate growth regulators is highly effective for micropropagation of *Centella asiatica*.

The regenerated plantlets showed good rooting and high survival rates during acclimatization, indicating their physiological stability. Similar results have been reported where *in vitro* raised plantlets of *Centella asiatica* exhibited genetic and biochemical stability after acc limatization.

Overall, the study confirms that *in vitro* propagation is an efficient and reliable method for mass multiplication of *Centella asiatica*. The optimal treatment identified was 3.0 mg/L BAP + 3.0 mg/L IAA, which produced maximum shoot proliferation, elongation, and rooting. This is consistent with previous research highlighting the importance of balanced growth regulator combinations for large-scale propagation and conservation of medicinal plants.

Table 4. Effect of different concentrations of BAP and IAA on In Vitro shoot initiation, proliferation, elongation and rooting of Centella asiatica.

S.No.	BAP Concentration (mg/L)	IAA Concentration (Mg/L)	7Day Initiation	14 day Shoot Proliferation	21 day Elongation	28day Rooting Status
1.	0.0	0.0	0%	0%	0%	No response
2.	1.0	1.0	45%	55%	60 %	Very poor rooting weak
3.	2.0	2.0	65%	75%	90%	Moderate rooting
4.	3.0	3.0	85%	95%	90%	Excellent rooting. Healthy plant
5.	4.0	4.0	50%	60%	55%	Callus formation, poor Rooting

Figure 1: In Vitro regeneration of Centella asiatica: shoot proliferation and root induction under optimized culture conditions.

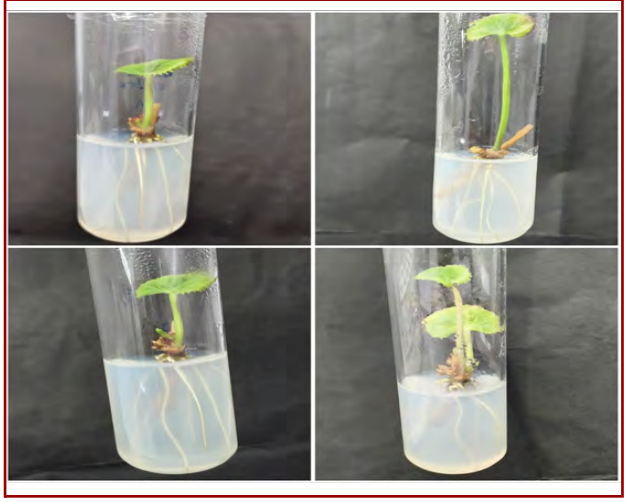

Figure 2: Ex Vitro acclimatization of micropropagated Centella asiatica plantlets under greenhouse conditions

CONCLUSION

The present study successfully demonstrates that *in vitro* propagation of *Centella asiatica* is highly influenced by the concentration and combination of plant growth regulators (PGRs), specifically BAP (Benzylaminopurine) and IAA (Indole-3-acetic acid). The results clearly show that an exogenous supply of PGRs is essential for promoting growth and development, as the hormone-free medium failed to induce any significant morphological changes. Among the different explant types, nodal explants proved to be the most responsive, likely due to the presence of pre-existing meristems.

The optimal combination of 3.0 mg/L BAP and 3.0 mg/L IAA yielded the best results, with the highest percentages of initiation, shoot proliferation, elongation, and excellent rooting. This aligns with earlier studies that emphasized the role of balanced cytokinin and auxin levels for enhancing shoot multiplication and plant regeneration in *Centella asiatica*. Lower concentrations of 2.0 mg/L BAP and 2.0 mg/L IAA also provided satisfactory results, confirming that hormonal balance is crucial for coordinating both shoot and root development. However, higher concentrations of PGRs (4.0 mg/L BAP + 4.0 mg/L IAA) resulted in a decline in growth performance, with decreased elongation and callus formation, supporting the concept that excessive hormone levels can disrupt plant development.

Surface sterilization, utilizing Bavistin and $HgCl_2$, effectively reduced contamination rates, further highlighting the importance of proper sterilization protocols in the success of *in vitro* cultures. The use of Murashige and Skoog (MS) medium provided essential nutrients, contributing to the successful

growth and development of explants, and subsequent acclimatization of regenerated plantlets showed high survival rates and physiological stability.

In conclusion, the study affirms that *in vitro* propagation of *Centella asiatica* is a reliable method for large-scale multiplication, and the optimal PGR combination of 3.0 mg/L BAP + 3.0 mg/L IAA is recommended for efficient propagation and conservation of this medicinal plant species.

Conflict of interest: There is no conflict of interest.

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Optimization of Surface Sterilization and Plant Growth Regulator Regimes for *In vitro* Micropropagation of *Dendrocalamus stocksii* (Munro) Benth.ex Gamble via Nodal Segment Culture

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ABSTRACT:

Dendrocalamus stocksii (Munro) Benth.ex Gamble is an ecologically and industrially important sympodial bamboo species distributed across the Western Ghats of India, valued for its structural strength and diverse commercial applications. Conventional propagation of this species is severely constrained by the gregarious flowering habit, low seed viability, and limited efficiency of vegetative methods, necessitating the development of reliable *in vitro* propagation protocols. The present study was undertaken to optimize surface sterilization procedures and plant growth regulator (PGR) regimes for the micropropagation of D. stocksii using nodal segment explants collected from the Bamboosetum, State Forest Research Institute, Jabalpur, Madhya Pradesh, India. Surface sterilization experiments demonstrated that sequential treatment with 1.0% Bavistin (carbendazim) for 30 minutes followed by 0.10% mercuric chloride (HgCl2) for 10 minutes yielded the minimum contamination rate of 3% and maximum explant survival of 90%. For shoot proliferation, nodal segments were cultured on Murashige and Skoog (MS) basal medium supplemented with varying concentrations of 6-Benzylaminopurine (BAP; 0.0–4.0 mg/L) in combination with Naphthalene Acetic Acid (NAA; 0.0–0.5 mg/L). The treatment comprising 3.0 mg/L BAP + 0.5 mg/L NAA proved optimal, producing the highest shoot initiation (85–90%), proliferation (90–95%), and elongation (90%) at 7, 14, and 21 days post-inoculation, respectively, with well-developed, healthy plantlets recorded at 28 days. Supraoptimal BAP concentrations (4.0 mg/L) resulted in marked growth inhibition and callus formation. The protocols established in this study provide a reproducible scientific foundation for the large-scale clonal propagation and conservation of D. stocksii.

KEY WORDS: *Dendrocalamus Stocksii*, Micropropagation, Nodal Segment Culture, BAP, NAA, Surface Sterilization, Shoot Proliferation, Plant Tissue Culture.

INTRODUCTION

Bamboo, a member of the family Poaceae and subfamily Bambusoideae, represents one of the most ecologically and economically significant plant groups in the world. Comprising over 1,500 species distributed across approximately 50 genera, bamboo occupies diverse ecological niches spanning tropical, subtropical, and temperate regions of Asia, Africa, and the Americas [26,12]. Its remarkable biological attributes including an exceptionally rapid growth rate, high biomass productivity, and a renewable harvest cycle have earned it the designation of "green gold" among plant resources [22] Beyond its economic utility, bamboo fulfils critical ecological functions: it sequesters carbon at rates comparable to fast-growing tree species, stabilises degraded soils, regulates local hydrology, and supports diverse faunal communities within bamboo-dominant ecosystems[15,9].

Dendrocalamus stocksii (Munro) Benth.ex Gamble is a clump-forming, sympodial bamboo species of considerable ecological and industrial importance. Naturally distributed across the tropical and sub-tropical forests of the Central and Western Ghats of India encompassing the states of Maharashtra, Goa, Karnataka, and Kerala it is known regionally by vernacular names including Marihal, Manga, Men, and Chiva [23]. The species is characterised by its large, straight, thick-walled culms attaining heights of 10–20 metres and diameters of 5–10 cm, properties that render it particularly well-suited for construction, scaffolding, furniture fabrication, and handicraft industries [24]. Its adaptability to diverse soil types, high biomass yield, and structural integrity position D. stocksii as a priority species for commercial cultivation, agroforestry integration, and ecological restoration programmes in peninsular India.

Despite its multifaceted significance, the large-scale deployment of *Dendrocalamus stocksii* in plantation and conservation programmes is severely constrained by the inherent limitations of conventional propagation methods. Seed-based propagation is fundamentally unreliable owing to the species' gregarious, mast-seeding reproductive strategy a phenomenon wherein bamboo populations flower synchronously, set seed, and subsequently die at intervals of several decades [10,7]. The infrequency and unpredictability of flowering events, combined with the characteristically low viability and rapid loss of germinability of bamboo seed, render seed propagation practically unfeasible for sustained nursery production [20]. Vegetative propagation techniques, including rhizome division and branch or culm cuttings, are constrained by the limited availability of suitable propagules, the low rooting efficiency of mature tissue, the labour and resource costs associated with large-scale operations, and the inherent risk of pathogen transmission from source plants to propagated material [2].

Plant tissue culture and specifically micropropagation offers a scientifically robust and practically viable alternative for the rapid, year-round, large-scale clonal multiplication of elite bamboo genotypes under aseptic laboratory conditions. Micropropagation bypasses the limitations of conventional methods by enabling the regeneration of genetically uniform, pathogen-free plantlets from meristematic explant sources, independent of seasonal constraints [13,3]. The nodal segment, bearing an axillary meristem of established organogenic competence, has been established as the preferred explant type for shoot multiplication in recalcitrant woody monocots, including numerous bamboo species [19,1]. The successful deployment of micropropagation protocols for bamboo species, however, is contingent upon the careful optimization of two rate-limiting parameters: the elimination of microbial contamination through effective explant surface sterilization, and the identification of appropriate plant growth regulator (PGR) concentrations and combinations that reliably promote shoot proliferation and subsequent root induction.

Plant growth regulators occupy a central position in the regulation of morphogenesis in plant tissue culture systems. Cytokinins, and particularly 6-Benzylaminopurine (BAP), are the most widely employed class of PGRs for the stimulation of axillary bud break and multiple shoot proliferation in bamboo micropropagation, functioning by overcoming apical dominance and activating latent axillary meristems [25,21. The concentration of BAP must be empirically optimized for each species, as both sub-optimal and supra-optimal concentrations impair shoot induction, with excessive cytokinin levels frequently associated with vitrification, abnormal morphology, and callus formation at the expense of organised shoot development [14]. Auxins, including Naphthalene Acetic Acid (NAA) and Indole-3-butyric acid (IBA), complement cytokinin activity by promoting root initiation in regenerated shoots and maintaining cellular polarity during organogenesis [27]. The judicious combination and sequential application of cytokinins and auxins across distinct culture stages constitutes the methodological cornerstone of successful bamboo micropropagation protocols.

Successful micropropagation protocols have previously been established for a number of commercially important bamboo species, including Bambusa bambos [1], Dendrocalamus asper [17], Bambus abalcooa [4], and Dendrocalamus giganteus [16], confirming the general applicability of cytokinin-auxin-based tissue culture systems to this plant group. However, species-specific variation in explant responsiveness, optimal PGR concentrations, and rooting behaviour necessitates independent protocol development for each target species. To date, no systematically optimized micropropagation protocol has been reported for *Dendrocalamus stocksii*, representing a significant gap in the literature that limits the conservation and commercial exploitation of this priority species.

The present investigation was therefore designed to develop and optimize an *in vitro* propagation protocol for *Dendrocalamus stocksii* using nodal segment explants sourced from the Bamboosetum maintained at the State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India. The study was specifically directed towards achieving the following objectives:

1. To evaluate the effect of different plant growth regulators (PGRs), with emphasis on varying concentrations of BAP in combination with NAA, on axillary bud break and shoot proliferation in *Dendrocalamus stocksii* under *in vitro* conditions.
2. To optimize surface sterilization treatments by assessing the efficacy of Bavistin (carbendazim) and mercuric chloride (HgCl2) in minimizing microbial contamination while preserving explant viability.

The findings of this study was expected to contribute to the growing body of knowledge on bamboo tissue culture and provide a reproducible, evidence-based foundation for the large-scale clonal propagation of D. stocksii in support of plantation forestry, agroforestry, and conservation programmes in India.

MATERIALS AND METHODS

Plant Material and Explant Collection: Healthy, phenotypically superior plants of *Dendrocalamus stocksii* (Munro) Benth.ex Gamble were selected as the source of explant material from the Bamboosetum maintained under the Biotechnology Division, State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India. Nodal segments bearing intact axillary buds were chosen as the explant type on account of their established high regenerative and morphogenic competence in monocotyledonous tissue culture systems (Ramanayake et al., 2001). Explants of uniform size (approximately 2–3 cm in length) were excised from actively growing, young shoots using sterile sharp secateurs and immediately transported to the laboratory in sealed polythene bags lined with moistened sterile filter paper to minimize desiccation stress and preserve tissue viability. To ensure optimal explant responsiveness, collection and inoculation were performed on the same day, thus minimizing the interval between excision and culture establishment.

Laboratory Infrastructure and Equipment: All experimental procedures were carried out in the dedicated Plant Tissue Culture Laboratory, Biotechnology Division, State Forest Research Institute, Jabalpur. Standard laboratory glassware including conical flasks, beakers, volumetric flasks, measuring cylinders, pipettes, Petri dishes, culture tubes, and test tube stands were also employed throughout. Sterile stainless-steel forceps, scalpels fitted with size 22 surgical blades, and scissors were used for all explant manipulation and trimming procedures under aseptic conditions.

2.3 Surface Sterilization of Explants: Effective surface sterilization, balancing the elimination of microbial contaminants with the preservation of explant viability, is the foundational prerequisite for successful *in vitro* culture establishment [21]. A sequential, multi-agent sterilization protocol was developed and optimized for nodal segment explants of D. stocksii as detailed below.

Pre-wash: Freshly collected nodal segments were washed thoroughly under running tap water for 20–30 minutes to remove gross surface particulates, dust, and loosely adhering epiphytic microorganisms.

Detergent treatment: Explants were immersed in a dilute commercial detergent solution (Extran, 2–3 drops per 100 mL sterile distilled water) for 10 minutes with gentle agitation, followed by rinsing with sterile double-distilled water (DDW) three to four times to remove detergent residues.

Table 2.2: Sequential surface sterilization protocol for nodal segment explants of *Dendrocalamus stocksii*

Step	Agent	Concentration	Duration	Purpose
1	Tap water wash	—	20–30 min	Removal of gross surface contaminants
2	Detergent (Extran)	Dilute (2–3 drops/100 mL)	10 min	Reduction of surface microbial load
3	Sterile DDW rinse	—	3–4 rinses	Removal of detergent residue
4	Bavistin (carbendazim)	0.0–1.0% (w/v)	25–30 min	Antifungal surface treatment
5	Sterile DDW rinse	—	3–4 rinses	Removal of Bavistin residue
6	Ethanol	70% (v/v)	30–60 sec	Broad surface disinfection
7	Mercuric chloride ($HgCl_2$)	0.01–0.10% (w/v)	5–10 min	Broad-spectrum surface sterilization
8	Sterile DDW rinse	—	4–5 rinses	Complete removal of $HgCl_2$

Fungicide treatment (Bavistin): To reduce surface fungal load, explants were treated with aqueous solutions of Bavistin (carbendazim; BASF, India) at varying concentrations (0.0%, 0.1%, 0.2%, 0.6%, 0.8%, and 1.0% w/v) for exposure durations of 25–30 minutes on an orbital shaker. Solutions were prepared by dissolving the requisite quantity of Bavistin in sterile double-distilled water. Following treatment, explants were rinsed with sterile DDW three to four times.

Mercuric chloride ($HgCl_2$) treatment: Explants were subsequently surface-sterilized by immersion in aqueous solutions of mercuric chloride ($HgCl_2$) at concentrations of 0.01%, 0.02%, 0.05%, and 0.10% (w/v) for 5–10 minutes under continuous agitation. Mercuric chloride functions as a potent broad-spectrum biocide effective against both bacterial and fungal contaminants through disruption of cell membrane integrity and enzyme inactivation [11].

Final rinse: Following $HgCl_2$ treatment, all explants were rinsed exhaustively four to five times with sterile double-distilled water inside the laminar air flow cabinet to ensure complete removal of residual sterilant prior to inoculation.The complete sterilization protocol is summarized in Table 2.2.

Table 2.4: Stock solution compositions for MS medium preparation

Stock	Components	Concentration
Stock I — Macronutrients (20×)	NH_4NO_3, KNO_3, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4	20× concentrated
Stock II — Micronutrients (200×)	KI, H_3BO_3, $MnSO_4$, $ZnSO_4$, Na_2MoO_4, $CuSO_4$, $CoCl_2$	200× concentrated
Stock III — Iron (200×)	$FeSO_4 \cdot 7H_2O$, $Na_2EDTA \cdot 2H_2O$	200× concentrated
Stock IV — Vitamins (200×)	myo-Inositol, Nicotinic acid, Pyridoxine, Thiamine, Glycine	200× concentrated

Culture Medium Composition and Preparation: Murashige and Skoog (MS) basal medium [11] was used as the standard nutrient formulation for all *in vitro* propagation experiments, as it provides a comprehensive supply of macronutrients, micronutrients, vitamins, and organic supplements required for optimal *in vitro* growth of plant tissues.

Preparation of Stock Solutions: To facilitate accurate and efficient medium preparation, concentrated stock solutions were prepared for macronutrients (Stock I, 20×), micronutrients (Stock II, 200×), iron (Stock III, 200×), and vitamins (Stock IV, 200×) as detailed in Table 2.4. Stock solutions were stored at 4°C in amber glass bottles and used within four weeks of preparation.

Medium Preparation Procedure: For the preparation of one litre of MS medium, 500 mL of double-distilled water was taken in a conical flask and 30 g of sucrose was added and dissolved completely with stirring. Stock solutions were added sequentially: 50 mL of Stock I, 5 mL each of Stocks II, III, and IV. The required volume of plant growth regulator (PGR) stock solution was then incorporated. The volume was made up to 1,000 mL with sterile DDW and the pH adjusted to 5.7 ± 0.1 using 1N HCl or 1N NaOH as required, verified with a calibrated digital pH meter. Agar (8 g/L) was then added and dissolved by heating in a microwave oven until a clear, homogeneous solution was obtained. The medium was dispensed (approximately 15–20 mL) into pre-cleaned borosilicate culture tubes or conical flasks, sealed with cotton plugs or polypropylene caps, and sterilized by autoclaving at 121°C and 15 psi for 20–30 minutes (moist heat sterilization). Heat-sensitive PGRs such as BAP and NAA were filter-sterilized through 0.22 µm membrane filters and added aseptically to the autoclaved, cooled medium (approximately 50°C) prior to dispensing. Sterilized media were allowed to solidify at room temperature and stored at 4°C until use.

Plant Growth Regulators (PGRs): Plant growth regulators were incorporated into the MS basal medium to investigate their effects on shoot proliferation and root induction. The cytokinins and auxins employed, along with their concentration and different ranges.

Stock solutions of BAP and NAA were prepared by dissolving the required quantity in a minimum volume of 1N NaOH, followed by dilution to the target concentration with double-distilled water. NAA stock solutions were prepared by dissolving in a minimum volume of 95% ethanol before diluting with DDW. All PGR stock solutions were stored at 4°C and filter-sterilized prior to addition to autoclaved medium.

Inoculation and Culture Conditions: All inoculation procedures were performed within the laminar air flow (LAF) cabinet, which was decontaminated by exposure to UV irradiation for 20 minutes prior to each session and wiped thoroughly with 70% ethanol. Forceps, scalpels, and scissors were sterilized by immersion in 70% ethanol and flaming between operations on different explants to prevent cross-contamination. Surface-sterilized nodal segments were trimmed at both cut ends using a sterile scalpel to remove damaged or discoloured tissue, and inoculated vertically into culture tubes with the basal end embedded 5–8 mm into the solidified medium. Culture vessels were sealed with Parafilm

or polypropylene caps and labelled with treatment details and date of inoculation.

Inoculated cultures were transferred immediately to a controlled-environment culture room maintained at 25 ± 2°C under a 16-hour photoperiod (2,000–3,000 lux light intensity provided by cool-white fluorescent lamps) and 8 hours of darkness, with relative humidity maintained at 60–70%. Cultures were examined at regular intervals for evidence of microbial contamination, bud break, callus formation, shoot initiation, and root development.

Subculturing: Subculturing was performed at intervals of three to four weeks to sustain shoot proliferation and transfer developing shoots to fresh medium. Prior to each subculture session, the LAF cabinet was UV-irradiated for 20 minutes and wiped with 70% ethanol. Instruments were re-sterilized using a hot-bead sterilizer (250°C, 15 seconds) between transfers. Shoots were excised at the nodal region using sterile scalpels and transferred individually to freshly prepared medium of the appropriate composition. Growth parameters including shoot initiation percentage, shoot proliferation percentage, shoot elongation (cm), and rooting response were recorded at intervals of 7, 14, 21, and 28 days post-inoculation.

Hardening and Acclimatization: Well-developed, rooted plantlets were carefully removed from culture vessels and the adhering agar was washed off completely under running water to prevent fungal proliferation in the substrate. Plantlets were transferred to a soil mixture composed of garden soil, cocopeat, and vermiculite (1:1:1, v/v/v) in plastic pots. Pots were maintained initially in a humidity chamber under controlled conditions (high humidity, 25 ± 2°C, diffuse light) to minimize transplant shock, and relative humidity was progressively reduced over a period of two to three weeks to acclimatize the plantlets to ambient ex vitro conditions. The survival percentage and growth vigour of hardened plantlets were recorded at weekly intervals.

Experimental Design and Statistical Analysis: All experiments were conducted in a completely randomized design (CRD) with a minimum of three replicates per treatment, each replicate comprising five culture tubes. Data are expressed as mean ± standard error (SE). One-way Analysis of Variance (ANOVA) was applied to assess the statistical significance of differences among treatment groups, followed by Duncan's Multiple Range Test (DMRT) for post-hoc pairwise comparisons at a significance level of $p \leq 0.05$. All statistical analyses were performed using SPSS software (version 26.0, IBM Corp.).

RESULTS AND DISCUSSION

The present investigation evaluated the effect of varying concentrations and combinations of plant growth regulators (PGRs) on *in vitro* shoot initiation, proliferation, elongation, and plantlet development in *Dendrocalamus stocksii*, alongside the optimization of surface sterilization protocols using Bavistin (carbendazim) and mercuric chloride (HgCl2). Observations were recorded at regular intervals of 7, 14, 21, and 28 days post-inoculation. The results are presented and discussed under the following sub-sections.

3.1 Experiment I: Optimization of Surface Sterilization Protocols

Effect of Bavistin (Carbendazim) Concentration and Exposure Duration on Contamination Rate: Surface sterilization constitutes the most critical preliminary step in the establishment of axenic plant tissue cultures, as field-collected explants of woody monocots such as *Dendrocalamus stocksii* invariably harbour a substantial load of fungal endophytes, surface bacteria, and airborne spores that are refractory to simple washing procedures (Bhojwani and Razdan, 1996). In the present study, nodal segment explants were treated with Bavistin (carbendazim) solutions across a concentration gradient of 0.0–1.0% (w/v) for exposure durations of 5–35 minutes. The resultant contamination rates are presented in Table 3.1.

Table 3.1: Effect of Bavistin concentration and exposure duration on contamination rate in nodal segment explants of *Dendrocalamus stocksii*

Treatment	Bavistin Concentration (% w/v)	Duration (min)	Contamination Rate (%)
T0	0.0 (Control)	5	90
T1	0.1	10	70
T2	0.2	15	50
T3	0.6	20	30
T4	1.0	30	3
T5	0.8	35	15

A pronounced concentration- and duration-dependent reduction in contamination rate was observed across the Bavistin treatment series (Table 3.1). The untreated control (T0; 0.0% Bavistin, 5 minutes) exhibited the highest contamination rate of 90%, confirming that surface washing alone is inadequate for the elimination of fungal and bacterial contaminants from bamboo nodal explants. Contamination rates declined progressively with increasing Bavistin concentration and exposure duration, from 70% in T1 (0.1%, 10 min) to 50% in T2 (0.2%, 15 min) and 30% in T3 (0.6%, 20 min). The minimum contamination rate of 3% was recorded in T4 (1.0% Bavistin, 30 min), establishing this as the most effective fungicidal treatment among those evaluated.

Notably, T5 (0.8% Bavistin, 35 min) yielded a higher contamination rate (15%) than T4, despite its longer exposure duration. This apparent anomaly may be attributable to the

sub-optimal fungicidal concentration of 0.8% relative to 1.0%, confirming that concentration is the primary determinant of Bavistin efficacy over exposure time within the ranges tested. Carbendazim, the active systemic benzimidazole component of Bavistin, exerts its antifungal action through inhibition of fungal tubulin polymerization, thereby disrupting mitosis and cell division in sensitive fungal species [5]. Its systemic mode of action renders it particularly effective against endophytic fungi that are inaccessible to surface-acting agents. Comparable findings have been reported by [1] in Bambusa bambos and by [20] in Bambusa vulgaris, wherein Bavistin treatment at concentrations of 0.5–1.0% significantly reduced fungal contamination in bamboo nodal cultures. The treatment T4 (1.0% Bavistin, 30 minutes) is therefore recommended as the optimal fungicidal sterilization step for D. stocksii nodal explants prior to HgCl2 treatment.

Effect of Mercuric Chloride ($HgCl_2$) Concentration and Exposure Duration on Explant Survival: Following Bavistin pre-treatment, explants were subjected to mercuric chloride (HgCl2) solutions at concentrations of 0.01–0.10% (w/v) for durations of 2–10 minutes to achieve complete broad-spectrum surface decontamination. Explant survival rates under each treatment are presented in Table 3.2.

Table 3.2: Effect of HgCl2 concentration and exposure duration on explant survival in *Dendrocalamus stocksii* nodal segments

Treatment	$HgCl_2$ Concentration (% w/v)	Duration (min)	Survival (%)	Observation
T1	0.01	2	60	High contamination
T2	0.02	3	70	Moderate sterilization
T3	0.05	5	50	Tissue damage observed
T4	0.10	10	90	Best sterilization, maximum survival

The results demonstrated a clear relationship between $HgCl_2$ concentration, exposure duration, and explant survival (Table 3.2). At the lowest concentration of 0.01% $HgCl_2$ for 2 minutes (T1), a survival rate of only 60% was recorded, accompanied by persistent high contamination, indicating insufficient sterilization efficacy. Moderate sterilization was achieved at 0.02% for 3 minutes (T2), yielding 70% survival. Treatment T3 (0.05%, 5 minutes) produced an unexpectedly lower survival rate of 50%, likely attributable to the onset of phytotoxic tissue damage at this concentration-duration combination, consistent with the known cytotoxic properties of mercuric ions at intermediate exposure thresholds [11].

The highest explant survival of 90% with minimum contamination was recorded in T4 (0.10% $HgCl_2$, 10 minutes), establishing this treatment as the optimal $HgCl_2$ sterilization condition for D. stocksii nodal segments. Mercuric chloride exerts its biocidal action through the non-specific precipitation of cellular proteins and inactivation of sulfhydryl-containing enzymes in microbial cells, making it one of the most potent broad-spectrum surface sterilants available for plant tissue culture applications [8]. The efficacy of 0.10% HgCl2 at 5–10 minutes in bamboo tissue culture has similarly been reported by [15] in Bambusa balcooa and by [15] in Dendrocalamus giganteus, lending strong comparative support to the present findings. The superior outcome obtained at T4 relative to T3 further demonstrates that, within species-specific tolerance thresholds, a higher concentration of HgCl2 combined with an adequate exposure period achieves decontamination without proportionally increasing phytotoxic injury, provided that thorough post-treatment rinsing with sterile distilled water is performed.

The combined sequential protocol of 1.0% Bavistin for 30 minutes followed by 0.10% $HgCl_2$ for 10 minutes, with exhaustive rinsing after each step, is thus recommended as the optimized surface sterilization protocol for nodal segment explants of D. stocksii.

3.2 Experiment II: Effect of BAP and NAA on *In Vitro* Shoot Initiation, Proliferation, Elongation, and Plantlet Development *In vitro*-raised nodal segments of D. stocksii were cultured on MS basal medium supplemented with varying concentrations of BAP (0.0–4.0 mg/L) in combination with NAA (0.0–0.5 mg/L). Growth responses were recorded at 7, 14, 21, and 28 days post-inoculation to capture the successive stages of shoot initiation, proliferation, elongation, and final plantlet development, respectively. The consolidated data are presented in Table 3.3.

Effect on Shoot Initiation (7 Days): Shoot initiation, defined as the visible emergence and elongation of an axillary bud from the nodal explant, was recorded at 7 days post-inoculation. A well-defined concentration-dependent pattern of response was observed across the BAP–NAA treatment series (Table 3.3). In the hormone-free control (T0), a markedly low shoot initiation of 20–30% was recorded, confirming that endogenous hormonal levels within the explant tissue were insufficient to overcome axillary bud dormancy under *in vitro* conditions. The poor initiation response in the absence of exogenous PGRs is

consistent with the reported high cytokinin requirement for activation of dormant axillary meristems in bamboo species [14,21,1].

Shoot initiation improved progressively with increasing BAP concentration, from 45% in T1 (1.0 mg/L BAP + 0.2 mg/L NAA) to 65% in T2 (2.0 mg/L BAP + 0.3 mg/L NAA). The maximum shoot initiation of 85–90% was recorded in T3 (3.0 mg/L BAP + 0.5 mg/L NAA), demonstrating that this PGR combination most effectively released axillary buds from dormancy. The stimulatory effect of BAP on bud break is attributable to its role in promoting cytokinin-regulated transcription of cell cycle genes, specifically those governing the G1-to-S phase transition, thereby activating meristematic cells that are arrested under apical dominance [25]; [27]. The supplementary presence of NAA at 0.5 mg/L likely contributed to initiation by modulating auxin-cytokinin crosstalk and facilitating vascular differentiation at the base of emerging shoots.

Table 3.3: Effect of BAP and NAA concentrations on in vitro growth parameters of *Dendrocalamus stocksii* at successive observation intervals

Treatment	BAP (mg/L)	NAA (mg/L)	7 Days Initiation (%)	14Days Proliferation (%)	21 Days Elongation (%)	28 Days Final Status
T0	0.0	0.0	20–30	25–35	30–40	Very poor growth
T1	1.0	0.2	45	55–60	60–65	Weak shooting
T2	2.0	0.3	65	75–80	85–90	Moderate shooting
T3	3.0	0.5	85–90	90–95	90	Excellent shooting; healthy plantlets
T4	4.0	0.5	50–60	55–65	60	Callus formation; poor growth

At the supraoptimal concentration of 4.0 mg/L BAP (T4), shoot initiation declined substantially to 50–60%, consistent with the well-established phenomenon of cytokinin toxicity at elevated concentrations, wherein excessive BAP disrupts hormonal homeostasis, induces oxidative stress, and suppresses normal organogenesis in favour of unorganized callus proliferation [14]. Similar inhibitory responses to high BAP concentrations have been reported in Dendrocalamus asper [17]and Bambusa bambos [1], strongly corroborating the present findings.

Effect on Shoot Proliferation (14 Days): Shoot proliferation, assessed as the percentage of explants producing multiple shoots per nodal segment, was recorded at 14 days post-inoculation. The proliferation data broadly paralleled the initiation response, with a pronounced concentration-dependent optimum at 3.0 mg/L BAP (Table 3.3). The control (T0) recorded a very low proliferation of 25–35%, confirming the essential role of exogenous cytokinins in driving cell division and multiple shoot formation under *in vitro* conditions. Proliferation increased substantially from 55–60% in T1 (1.0 mg/L BAP) to 75–80% in T2 (2.0 mg/L BAP), with the maximum proliferation of 90–95% achieved in T3 (3.0 mg/L BAP + 0.5 mg/L NAA). This treatment consistently produced multiple shoots per explant, with vigorous axillary bud activation and compact, well-organized shoot clusters.

The superior performance of T3 reflects the synergistic interaction between BAP-mediated cytokinin signalling and NAA-mediated auxin activity: while BAP drives cell division and lateral bud release, NAA at low concentrations facilitates shoot vascularization and maintains meristematic cell competence without suppressing shoot organogenesis [6]. Comparable proliferation rates at 2.5–3.0 mg/L BAP have been reported in Bambusa balcooa by [15], in Dendrocalamus strictus by Pattnaik and Chand (1996), and in Dendrocalamus giganteus by [15], confirming that 3.0 mg/L BAP represents a broadly optimal cytokinin concentration for multiple shoot induction in tropical bamboo species.

At T4 (4.0 mg/L BAP), proliferation declined markedly to 55–65% and was accompanied by the formation of abnormal, hyper-hydric callus tissue at the base of explants, indicative of the transition from organised organogenesis to unorganized somatic cell proliferation under conditions of hormonal excess [14]. This inhibitory response underscores the critical importance of maintaining BAP within species-specific optimal thresholds during the proliferation stage.

Effect on Shoot Elongation (21 Days): Shoot elongation, recorded at 21 days post-inoculation, represents a critical developmental transition from compact shoot clusters to individualized, elongated shoots of sufficient length for subsequent rooting or subculture. In the control (T0), elongation was very poor (30–40%), and shoots remained stunted and physiologically weak, consistent with the absence of exogenous growth hormone supplementation. Moderate elongation (60–65%) was observed at T1 (1.0 mg/L BAP + 0.2 mg/L NAA), which improved to 85–90% in T2 (2.0 mg/L BAP + 0.3 mg/L NAA). The highest elongation response of approximately 90% was recorded in T3 (3.0 mg/L BAP + 0.5 mg/L NAA), wherein shoots were well-elongated, turgid, and displayed normal leaf expansion.

The positive effect of low-concentration NAA on shoot elongation in this study is consistent with the established role of auxins in promoting cell elongation through the acid growth mechanism, wherein auxin-induced proton pumping loosens the cell wall and facilitates turgor-driven cellular expansion (Taiz and Zeiger, 2010). The combination of cytokinin-driven cell division with auxin-promoted elongation in T3 thus produced the most morphologically advanced shoots at 21 days. At T4 (4.0 mg/L BAP), elongation declined to 60%, likely reflecting the known antagonistic effect of supraoptimal cytokinin concentrations on internode elongation, mediated through interference with the gibberellin signalling pathway responsible for cell elongation in shoot internodes (14 27].

Effect on Final Plantlet Development and Shooting (28 Days): At 28 days post-inoculation, the overall status of plantlet development encompassing shoot organisation, root initiation, and general physiological vigour was assessed for each treatment. In the hormone-free control (T0), no proper shooting or root development was observed and cultures exhibited minimal growth, confirming that auxin supplementation is essential for rhizogenesis and that endogenous auxin levels are insufficient to sustain organised plantlet development under *in vitro* conditions.

Weak, poorly rooted shoots were observed in T1 (1.0 mg/L BAP + 0.2 mg/L NAA), and moderate shooting with limited rooting was recorded in T2 (2.0 mg/L BAP + 0.3 mg/L NAA). The most favourable outcome was achieved in T3 (3.0 mg/L BAP + 0.5 mg/L NAA), which produced well-developed plantlets with healthy, well-organised shoot systems and initial root primordia, indicating readiness for transfer to dedicated rooting medium and subsequent hardening. These plantlets exhibited normal morphology, robust leaf development, and uniform growth vigour, confirming the suitability of this PGR regime for the complete *in vitro* establishment of D. stocksii. At the highest PGR concentration (T4: 4.0 mg/L BAP + 0.5 mg/L NAA), plantlet quality was markedly compromised, with callus formation at the nodal base, poor shoot organisation, and negligible root initiation. Excessive cytokinin concentrations are known to promote unorganized callus proliferation at the expense of organised shoot and root development, and may also induce epigenetic changes that reduce the regenerative competence of cultured tissue over successive subcultures [14, 8]. This result strongly argues against the use of BAP concentrations exceeding 3.0 mg/L in D. stocksii micropropagation. Effect of BAP and NAA Concentrations on *In vitro* Shoot Initiation, Proliferation, Elongation, and Plantlet Development in *Dendrocalamus stocksii* results shown in bellow mention table no 3.4.

Table 3.4: Effect of BAP and NAA Concentrations on In Vitro Shoot Initiation, Proliferation, Elongation, and Plantlet Development in Dendrocalamus stocksii at Successive Observation Intervals (7, 14, 21, and 28 Days Post-Inoculation) on MS Basal

BAP (mg/L)	NAA (mg/L)	7 Days Initiation (%)	14 Days Proliferation (%)	21 Days Elongation (%)	28 Days Shooting / Final Status
0.0	0.0	20–30%	25–35%	30–40%	Very poor growth
1.0	0.2	45%	55–60%	60–65%	Weak shooting
2.0	0.3	65%	75–80%	85–90%	Moderate shooting
3.0	0.5	85–90%	90–95%	90%	Excellent shooting, healthy plantlets □
4.0	0.5	50–60%	55–65%	60%	Callus formation, poor growth

□Optimal treatment combination. BAP = 6-Benzylaminopurine; NAA = Naphthaleneacetic acid. Values represent percentage response of explants under each treatment.

The findings the present studies were in concordance with a substantial body of literature on bamboo micropropagation. [20] reported optimal shoot multiplication at 2.0–3.0 mg/L BAP in Bambusa vulgaris; [4] identified 3.0 mg/L BAP as the optimal concentration in Bambusa balcooa; and [11] similarly reported maximum proliferation at 3.0 mg/L BAP in Dendrocalamus giganteus. The convergence of these findings with the present results confirms that 3.0 mg/L BAP represents a broadly applicable optimum for cytokinin-mediated axillary shoot proliferation in tropical sympodial bamboo species, and that the inclusion of a low-concentration auxin complement (0.5 mg/L NAA) significantly enhances both elongation and overall plantlet quality.

CONCLUSION

The present investigation successfully established an optimized *in vitro* micropropagation protocol for *Dendrocalamus stocksii*, encompassing both surface sterilization and plant growth regulator management across all critical developmental stages. With respect to surface sterilization, a sequential treatment protocol comprising 1.0% Bavistin (carbendazim) for 30

minutes followed by 0.10% $HgCl_2$ for 10 minutes, with exhaustive rinsing after each step, proved most effective. This combination reduced contamination to a minimum of 3% while maintaining maximum explant survival of 90%, and is hereby recommended as the standard sterilization protocol for nodal segment explants of D. stocksii.

For *in vitro* growth and development, MS basal medium supplemented with 3.0 mg/L BAP in combination with 0.5 mg/L NAA (Treatment T3) consistently yielded the most favourable outcomes across all four observation intervals. This treatment achieved maximum shoot initiation (85–90%) at 7 days, highest proliferation (90–95%) at 14 days, optimal elongation (approximately 90%) at 21 days, and produced well-organized, healthy plantlets with incipient root primordia at 28 days. Both sub-optimal PGR concentrations (T1, T2) and supraoptimal concentrations (T4) resulted in significantly inferior growth responses, with T4 additionally inducing undesirable callus formation and morphological abnormalities indicative of cytokinin toxicity.

The convergence of the present findings with published data from related bamboo species including Bambusa vulgaris, Bambusa balcooa, Dendrocalamus strictus, and Dendrocalamus giganteus strongly validates the reproducibility and broader applicability of this protocol within tropical sympodial bamboos. The synergistic interaction between BAP-mediated cytokinin signalling and low-concentration NAA-mediated auxin activity observed in T3 represents the critical hormonal balance underlying successful *in vitro* establishment of this species.

These results collectively provide a reliable, reproducible, and scalable micropropagation protocol for D. stocksii that can be adopted for large-scale clonal propagation, germplasm conservation, and commercial nursery production of this economically and ecologically significant bamboo species.

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In vitro Micropropagation and Conservation of African Baobab (*Adansonia digitata* Linn.): Optimized Effect of BAP on Shoot Proliferation and Auxin Treatments Root Induction through Nodal Segment in Madhya Pradesh, India

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ABSTRACT:

Adansonia digitata Linn. (African Baobab), a keystone multipurpose tree species of the family Bombacaceae, holds exceptional ecological, nutritional, and medicinal significance across sub-Saharan Africa and naturalized regions of the Indian subcontinent, including Madhya Pradesh, India. Growing anthropogenic pressures, habitat fragmentation, and inadequate natural regeneration have raised urgent conservation concerns, necessitating the development of reliable in vitro propagation protocols for this species. The present study investigated the micropropagation of *A. digitata* through nodal segment culture using Murashige and Skoog (MS) basal medium. In Experiment I, the effect of five concentrations of 6-Benzylaminopurine (BAP: 0.5, 1.0, 2.0, 3.0, and 5.0 mg/L) on axillary bud break and shoot proliferation was evaluated. Maximum bud break (100%), shoot number (3.22 shoots/explant), shoot length (1.32 cm), and leaf count (2.25 leaves/shoot) were recorded at 3.0 mg/L BAP at 15 days post-inoculation, establishing this as the optimal cytokinin concentration for shoot multiplication. In Experiment II, auxin-mediated rhizogenesis was assessed using Indole-3-butyric acid (IBA: 0.5, 1.0, and 2.0 mg/L) and Naphthalene acetic acid (NAA: 0.5 mg/L). The highest rooting response of 20% was recorded at 60 days in shoots treated with 2.0 mg/L IBA, though overall rooting remained recalcitrant across all treatments. Surface sterilization employing 2% Bavistin followed by 0.1% mercuric chloride (HgCl2) was optimized for effective decontamination of field-collected nodal segments. These findings provide a foundational protocol for the clonal multiplication and ex situ conservation of *A. digitata*, and underscore the need for further optimization of rhizogenesis strategies in this recalcitrant woody species.

KEY WORDS: *Adansonia digitata*, African Baobab, Micropropagation, Nodal Segment Culture, Bap, Iba, Shoot Proliferation, Root Induction, Plant Tissue Culture, Conservation.

INTRODUCTION

Adansonia digitata Linn., commonly designated as the African Baobab, is a large, deciduous tree belonging to the family Bombacaceae. The genus Adansonia comprises eight recognized species distributed across the African continent, Madagascar, and Australia, with *A. digitata* being the most widely distributed among them [3]. The genus epithet honours

Michel Adanson, the eighteenth-century French naturalist credited with providing the earliest scientific description of the species. In vernacular usage, the tree is known by numerous regional names that reflect its broad cultural footprint: 'monkey bread tree' in English derived from the documented feeding behavior of primates on its fruit [19] and alternatively as the 'upside-down tree,''cream of tartar tree,' and 'dead-rat tree,' all of which allude to its striking morphological characteristics. In the Indian subcontinent, where the species has been long naturalized, it is variously known as Gorakhamla or Gorakh chinch (Marathi), Brahma Mulika (Kannada), Enugulou da Chettu (Telugu), and Anaippuli (Tamil). Perhaps most evocatively, the species is universally acknowledged as the 'Tree of Life,' a designation that encapsulates the extraordinary ecological, nutritional, and socio-cultural value it confers upon the communities and ecosystems it inhabits.

Adansonia digitata is distinguished by a suite of remarkable morphological and physiological adaptations that render it uniquely resilient in the world's most challenging arid and semi-arid environments. The tree's expansive root system, high water-retention capacity, and fire-resistant fibrous bark enable it to flourish across a broad precipitation gradient of 100 to 1,000 mm of annual rainfall [1]. Its succulent trunk, which may attain a diameter of up to 10 metres, functions as an internal reservoir capable of storing thousands of litres of water, providing a critical resource buffer during prolonged droughts. The species is distributed throughout sub-Saharan Africa from the Sudano-Sahelian zone through the savannas of Eastern and Southern Africa and has been introduced to parts of South and Southeast Asia, the Arabian Peninsula, the Caribbean, and various Indian Ocean archipelagos through historical trade routes and human migration [21]. Within India, significant naturalized populations occur in the states of Madhya Pradesh, Gujarat, and Maharashtra, a distribution widely attributed to Arab traders and early African migrants.

Ecologically, *A. digitata* functions as a keystone species within the semi-arid African savanna, supporting complex webs of biodiversity. Its hollow trunks and high canopies provide nesting habitat for avian species, while its bark and wood support specialist insect communities including the Baobab Hawkmoth. The flowers of the Baobab are pollinated chiefly by nocturnal fruit bats, establishing an obligate ecological dependency [8]. The tree also contributes substantially to soil health: seasonal leaf litter enhances local concentrations of phosphorus, potassium, and magnesium, while the rhizosphere of the Baobab harbours specia lisedmycorrhizal associations and beneficial bacterial communities that improve surrounding soil fertility. These attributes collectively position *A. digitata* as an irreplaceable structural element of the ecosystems it occupies.

The ethnobotanical significance of *A. digitata* is virtually unparalleled among African flora. Every component of the tree leaves, bark, fruit pulp, seeds, and roots has been systematically utilized by human communities for nutritional, medicinal, and material purposes [8,18]. The fruit pulp is a nutritional powerhouse, containing approximately six times more Vitamin C than oranges and rich quantities of calcium, potassium, dietary fibre, and antioxidants [19]. The leaves constitute a significant dietary protein source, and the seeds yield high-value oil characterized by a well-balanced fatty acid profile. From a phytochemical perspective, the species elaborates a diverse array of secondary metabolites including procyanidins, flavonol glycosides, terpenoids, sterols, and organic acids which underpin its well-documented biological activities, among them antioxidant, anti-inflammatory, antimicrobial, and glycemic-regulatory effects [7] These properties have attracted considerable interest from the pharmaceutical, nutraceutical, and cosmetic industries, contributing to a projected global market value of approximately $101 million by 2026.

Beyond its biological and economic value, *A. digitata* presents a subject of exceptional scientific interest with respect to its longevity and growth dynamics. Radiocarbon dating of multiple wood sections has confirmed that the oldest known specimens are capable of exceeding 2,000 to 2,500 years in age, rendering them among the most ancient angiosperm individuals on Earth. Unlike most temperate trees, the Baobab does not produce reliable annual growth rings; instead, its fibrous, parenchyma-rich wood undergoes seasonal volumetric changes expanding during wet periods and contracting during drought rendering standard dendrochronological methods unreliable. The phenomenon of multiple trunks fusing into a single apparent individual further complicates age estimation but also reveals the species' extraordinary capacity for structural regeneration over ecological timescales.

Despite its historical classification as a species of 'Least Concern' by the International Union for Conservation of Nature (IUCN), emerging evidence from 2025 to 2026 has established that *A. digitata* faces increasingly severe localized threats, including the sudden structural collapse of ancient individuals attributed to climate-change-induced hydrological stress, accelerating habitat fragmentation from agricultural expansion, and overexploitation driven by global demand for its super food derivatives (IUCN, 2026). A critical deficit in natural regeneration has been recorded across multiple sub-Saharan regions, raising substantive concerns about the long-term viability of existing populations. Importantly, naturalized populations within India including those documented in Madhya Pradesh are similarly threatened by deforestation, land-use change, and the absence of systematic conservation or propagation programmes for this species [18].

Given this conservation urgency, the development of reliable *in vitro* propagation systems for *A. digitata* assumes considerable scientific and practical significance. Conventional propagation of the Baobab through seeds is hampered by hard seed coat dormancy, slow germination, and highly variable seedling growth, while vegetative propagation through stem cuttings has yielded inconsistent results due to the recalcitrant rooting behaviour characteristic of this species [11,12]. Plant tissue

culture, specifically micropropagation via nodal segment culture, offers a viable alternative that enables the rapid clonal multiplication of genetically superior or threatened individuals under controlled aseptic conditions. Central to the success of any micropropagation protocol are two critical determinants: (i) the optimization of surface sterilization procedures to eliminate microbial contamination without causing phytotoxic damage to the explant, and (ii) the identification of appropriate plant growth regulator (PGR) regimes particularly auxins and cytokininsthat effectively promote *in vitro* shoot proliferation and subsequent ex vitro root induction.

Surface sterilization of explant material constitutes the first and most critical step in establishing axenic *in vitro* cultures. Field-collected explants of woody perennial species such as *A. digitata* typically carry a high load of fungal endophytes, surface bacteria, and fungal spores that are not readily eliminated by conventional washing alone. The selection and optimization of an effective sterilization protocol balancing the concentration and duration of sterilant exposure against explant survival is therefore a prerequisite for successful culture establishment. Among the agents most widely employed for explant sterilization in woody trees, Bavistin (carbendazim; a systemic benzimidazole fungicide) and mercuric chloride (HgCl2; a potent broad-spectrum biocide) have been shown to confer effective decontamination, particularly when applied sequentially and at optimized exposure durations [4,15]. However, the optimal duration of sterilant application is highly species-specific and must be empirically determined to minimize explant necrosis while achieving the desired level of sterility. To date, no systematic study has characterized the optimal Bavistin and HgCl2 sterilization parameters for nodal segments of *A. digitata*, representing a key gap that the present study seeks to address.

Following successful shoot proliferation via cytokinin-supplemented media, the induction of adventitious roots in *A. digitata* remains the principal bottleneck limiting the development of a complete micropropagation protocol for this species. Auxinsthe primary class of phytohormones responsible for root initiation are routinely incorporated into rooting media to promote rhizogenesis in *in vitro*-raised shoots. Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA), and Indole-3-acetic acid (IAA) are the three most commonly employed auxins in plant tissue culture, each differing in its stability, transport characteristics, and efficacy across species and tissue types [19]. In addition to auxin concentration, the duration of auxin exposure has been recognized as a critical parameter in root induction protocols, particularly in recalcitrant woody species: a short-duration, high-concentration auxin pulse can often achieve superior rhizogenic responses compared to continuous low-level supplementation, as it promotes root primordium initiation without the phytotoxic effects associated with prolonged auxin exposure [19].

Optimal type of auxin and the effect of varying exposure intervals specifically 5, 15, 25, and 35 minutes on both *in vitro* shoot development and ex vitro root induction in *A. digitata* have not been previously investigated, constituting a further gap addressed by this study.

In light of the foregoing, the present investigation was undertaken to develop and optimize an *in vitro* propagation protocol for *A. digitata* using nodal segment explants collected from a documented medicinal garden population at the State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India. The study was designed to systematically address the primary methodological bottlenecks in Baobab micropropagation surface sterilization efficiency and auxin-mediated rhizogenesis within the framework of the following specific objectives:

1. To study the effect of different sterilization treatments specifically varying concentrations and exposure durations of Bavistin (carbendazim) and mercuric chloride (HgCl2) on the surface decontamination efficiency and survival rate of nodal segment explants of Adansoni adigitata under *in vitro* conditions.
2. To evaluate the effect of three auxin types Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA), and Indole-3-acetic acid (IAA) applied at four exposure time intervals (5, 15, 25, and 35 minutes) on *in vitro* shoot development and ex vitro root induction in *Adansonia digitata*, with the aim of identifying the auxin type and treatment duration most conducive to successful rhizogenesis.

The findings of this study are expected to contribute meaningfully to the growing body of literature on the *in vitro* propagation of recalcitrant tropical tree species and to provide a reproducible, evidence-based foundation for the large-scale clonal multiplication and conservation of *A. digitata* in India. Given the species' multifaceted ecological, nutritional, and economic importance and the increasing recognition of its vulnerability to climate-driven and anthropogenic threats the establishment of efficient micropropagation protocols for this 'Tree of Life' represents both a scientific priority and a conservation imperative.

MATERIALS AND METHODS

Plant Material and Explants Collection: Nodal segments of *Adansonia digitata* Linn. was collected from a healthy, mature mother plant maintained in the Medicinal Garden under the Forest Conservation Division, State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India. Young, actively growing nodal segments of 2–3 cm in length were selected as explants material, as juvenile tissue is known to exhibit superior morphogenic competence in *in vitro* culture systems. Collection was carried out exclusively in the early morning hours to minimize desiccation stress resulting from direct solar radiation. Each nodal segment was excised using sterile, sharp secateurs and immediately transported to the laboratory in sealed polythene bags lined with moist filter paper to preserve tissue viability. To ensure optimal explant

response, collection and inoculation were performed on the same day, thus minimizing the interval between excision and culture establishment.

Laboratory Infrastructure and Equipment: All experimental procedures were conducted in a dedicated plant tissue culture laboratory, Biotechnology Division, State Forest Research Institute equipped with the following instruments and apparatuses:

- **pH Meter:** Used for measurement and adjustment of culture medium pH using 1 N NaOH and 1 N HCl solutions.
- **Orbital Shaker:** Used for continuous mixing of chemical solutions at defined speeds (rpm) during stock preparation and explant surface sterilization.
- **Laminar Air Flow Cabinet:** Provided a HEPA-filtered, unidirectional airflow working zone for all aseptic inoculation and transfer procedures.
- **Microwave Oven:** Used for rapid and homogeneous dissolution of agar into liquid culture medium.
- **Micropipettes (range: 0.1–1000 µL):** Used for accurate volumetric transfer of plant growth regulators, antibiotic solutions, and vitamins.
- **Forceps and Scalpels:** Sterile, stainless-steel forceps and size 22 scalpel blades were used for explants manipulation, trimming, and transfer under aseptic conditions within the laminar air flow cabinet.

Culture Medium Composition and Preparation: Murashige and Skoog (MS) basal medium [16] was used as the standard nutrient formulation for all *in vitro* propagation experiments. The medium was supplemented with sucrose (30 g/L) as the primary carbon and energy source, and agar (7 g/L; Hi-Media, India) as the gelling agent. The pH of each medium formulation was adjusted to 5.7 ± 0.1 prior to autoclaving, using 1 N NaOH or 1 N HCl as required and verified with a calibrated digital pH meter. Media were sterilized by autoclaving at 121°C and 15 psi for 20 minutes and dispensed (approximately 15 mL per tube) into pre-sterilized borosilicate culture tubes under aseptic conditions inside the laminar air flow cabinet.

Plant growth regulators (PGRs) were incorporated into the basal medium at varying concentrations to evaluate their effects on shoot proliferation and root induction. The following PGRs were employed:

Table 2.1: Major components of MS culture medium used in the present study

Component	Concentration / Quantity	Function
Murashige& Skoog basal salts	Full strength (4.43 g/L)	Macro- and micronutrient supply
Sucrose	30 g/L	Carbon source and osmotic balance
Agar	8 g/L	Solidifying agent
myo-Inositol	100 mg/L	Vitamin / metabolic cofactor
Thiamine-HCl (B1)	0.1 mg/L	Vitamin supplement
Pyridoxine-HCl (B6)	0.5 mg/L	Vitamin supplement
Nicotinic acid	0.5 mg/L	Vitamin supplement
pH (adjusted)	5.7 ± 0.1	Nutrient availability and gel stability
Sterilization	121°C, 15 psi, 20 min	Elimination of microbial contaminants

Auxins: Auxins promote cell elongation, root initiation, and callus induction. Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) were used in concentration ranges of 0.01–10.0 mg/L. As PGRs are sparingly soluble in water, stock solutions of auxins were prepared by dissolving the required quantity in a minimal volume of 95% ethanol (for NAA and IBA) or 1 N NaOH (for IAA and 2,4-D), followed by dilution to the target volume with double-distilled water.

Cytokinins: Cytokinins promote cell division and axillary shoot proliferation. 6-Benzylaminopurine (BAP), Kinetin, and Thidiazuron (TDZ) were used at concentrations of 0.1–10.0 mg/L. Cytokinin stock solutions were prepared by dissolving the compound in a minimum volume of 1 N NaOH, then diluting with double-distilled water to the required concentration. Zeatin and isopentenyladenine (iP) were also evaluated in select experimental treatments.

Surface Sterilization of Explants: Effective surface sterilization is critical for the elimination of exogenous microbial contaminants without compromising explant viability. A sequential, multi-step sterilization protocol was developed and optimized for *A. digitata* nodal segments, as follows:

Pre-wash: Freshly collected nodal segments were rinsed under running tap water for 10–15 minutes to remove gross surface particulates, dust, and loose epiphytic microorganisms.

Ethanol rinse: Segments were surface-wiped and briefly dipped in 70% (v/v) ethanol (prepared by diluting 70 mL of absolute ethanol with 30 mL of double-distilled water) for 30 seconds with gentle agitation, then rinsed twice with sterile double-distilled water.

Fungicide treatment: Explants were treated with a 2% (w/v) aqueous solution of Bavistin (carbendazim; BASF, India) for 10 minutes on an orbital shaker to eliminate fungal spores. The solution was prepared by dissolving 2 g of Bavistin in 100 mL of sterile double-distilled water.

Mercuric chloride ($HgCl_2$) treatment: Explants were submerged in a 0.1% (w/v) aqueous solution of mercuric chloride (prepared by dissolving 0.1 g of $HgCl_2$ in 100 mL of sterile double-distilled water) for 5–8 minutes with continuous shaking. Mercuric chloride is a potent broad-spectrum surface sterilant effective against both bacteria and fungi.

Final rinse: Following $HgCl_2$ treatment, explants were rinsed three to five times with sterile double-distilled water inside the laminar air flow cabinet to remove all traces of the sterilant prior to inoculation.

Table 2.2: Surface sterilization protocol for *A. digitata* nodal segment explants

Step	Agent	Concentration	Duration	Purpose
1	Tap water wash	—	10–15 min	Removal of gross surface contaminants
2	Ethanol	70% (v/v)	30 sec	Broad surface disinfection
3	Bavistin (carbendazim)	2% (w/v)	10 min	Fungicidal treatment
4	Mercuric chloride (HgCl2)	0.1% (w/v)	5–8 min	Broad-spectrum sterilization
5	Sterile distilled water	—	3–5 rinses	Removal of residual sterilants

Inoculation and Culture Conditions: Following surface sterilization, explants were trimmed to remove damaged or discolored tissue using sterile scalpels and forceps. Inoculation was performed entirely within the laminar air flow cabinet, which was decontaminated by exposure to UV light for 20 minutes prior to use and wiped down with 70% ethanol before each session. Sterile forceps and scalpels were sterilized intermittently using a hot-bead sterilizer between operations on different culture vessels to prevent cross-contamination. Surface-sterilized nodal segments were placed vertically into culture tubes containing the respective MS medium formulation, with the proximal (basal) cut surface embedded approximately 5–8 mm into the gelled medium. Inoculated cultures were sealed with Parafilm and maintained in a plant growth chamber under a 16-hour photoperiod (light intensity: 2000–2500 lux, provided by cool-white fluorescent lamps) at a controlled temperature of 25 ± 2°C. Cultures were observed at regular intervals for evidence of contamination, callus formation, shoot initiation, and root development.

Sub-culturing: Sub-culturing was performed at intervals of three to four weeks to maintain the proliferating shoot cultures and to transfer them to fresh medium. Prior to each subculture session, the laminar air flow cabinet was irradiated with UV light for 20 minutes and subsequently wiped with 70% ethanol. Instruments including forceps and scalpels were sterilized using a hot-bead sterilizer (250°C, 15 seconds) between each transfer to prevent cross-contamination between culture vessels. Shoots were excised at the nodal region and transferred individually to freshly prepared medium. At each subculture interval, observations and measurements pertaining to shoot length, number of nodes per shoot, and rooting response were recorded.

Statistical Analysis: All experiments were conducted in triplicate, with a minimum of five culture tubes per treatment combination. Data are expressed as mean ± standard error (SE). One-way Analysis of Variance (ANOVA) was applied to evaluate the statistical significance of differences among treatment groups, followed by Duncan's Multiple Range Test (DMRT) for post-hoc pairwise comparisons. A probability level of $p \leq 0.05$ was considered statistically significant. All statistical computations were performed using SPSS (version 26.0) or an equivalent software package.

RESULTS AND DISCUSSION

The present study investigated the *in vitro* propagation potential of *Adansonia digitata* Linn. through nodal segment culture. Two independent experiments were conducted: (i) evaluation of the effect of varying concentrations of 6-Benzylaminopurine (BAP) on axillary bud break and shoot proliferation, and (ii) assessment of the effect of different concentrations of Indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA) on *in vitro* root induction from *in vitro*-raised shoots. The results of both experiments are presented and discussed in the following sub-sections.

Experiment I: Effect of BAP on Axillary Bud Break and Shoot Proliferation in Adansonia digitata: Nodal segments of *A. digitata* were inoculated onto Murashige and Skoog (MS) basal medium supplemented with five concentrations of BAP (0.5, 1.0, 2.0, 3.0, and 5.0 mg/L) along with a hormone-free control (T0). Cultures were maintained under standard growth conditions and observations on bud break response (%), number of shoots per explants, shoot length (cm), and numbers of leaves per shoot were recorded at 15 days post-inoculation.

All five BAP treatments elicited a measurable growth response, including the hormone-free control, confirming that the nodal explants possessed inherent morphogenic competence. The pooled data are summarized in Table 3.1.

Table 3.1: Effect of different concentrations of BAP on bud break and shoot proliferation in nodal segments of *Adansonia digitata* (observations recorded at 15 days post-inoculation; values represent treatment means)

S. No.	Treatment (BAP mg/L)	Bud Break Response (%)	Number of Shoots (Mean)	Shoot Length (cm)	Number of Leaves (Mean)
1	T0 — Control (0.0)	60	1.00	0.08	0.45
2	T1 (0.5)	82	1.32	0.11	0.66
3	T2 (1.0)	100	2.11	1.11	2.10
4	T3 (2.0)	100	2.40	1.21	2.14
5	T4 (3.0)	100	3.22	1.32	2.25
6	T5 (5.0)	77	1.11	0.45	0.59

Note: Control (T0) bud break and growth values represent basal endogenous response in hormone-free MS medium.

Bud Break Response (%): Kumar et al. (2011) observed peak shoot multiplication in medicinal tree species within the 2.5–3.5 Bud break, defined as the visible emergence and elongation of an axillary meristem from the nodal explant, was recorded as a percentage of responsive explants per treatment. A concentration-dependent pattern of bud break was observed across the BAP gradient (Table 3.1). The highest bud break response of 100% was recorded in treatments T2 (1.0 mg/L), T3 (2.0 mg/L), and T4 (3.0 mg/L), demonstrating that intermediate concentrations of BAP most effectively released axillary buds from apical dominance. The bud break response declined at sub-optimal and supra-optimal concentrations, with T1 (0.5 mg/L) yielding a response of 82% and T5 (5.0 mg/L) yielding the lowest response of 77% among the BAP-treated groups. The inhibitory effect observed at 5.0 mg/L BAP is consistent with the widely reported phenomenon of cytokinin toxicity at elevated concentrations, wherein excessive cytokinin levels can suppress morphogenesis by disrupting hormonal equilibrium and inducing vitrification or oxidative stress in plant tissue [15]. The progressive decline in bud break response beyond 3.0 mg/L thus reflects the existence of an optimal cytokinin threshold for this species, above which the promotive effects of BAP are negated.

Number of Shoots per Explant: The mean number of shoots per explant increased significantly with increasing BAP concentration up to 3.0 mg/L, beyond which a pronounced decline was observed (Table 3.1). The maximum shoot number of 3.22 was recorded in T4 (3.0 mg/L BAP), followed by T3 (2.0 mg/L) with 2.40 shoots, and T2 (1.0 mg/L) with 2.11 shoots. The minimum number of shoots among BAP-treated cultures was recorded in T5 (5.0 mg/L) with 1.11 shoots per explants, a value comparable to the hormone-free control (T0: 1.00 shoot). The control treatment, while recording low shoot proliferation as expected, confirmed that some degree of endogenous cytokinin activity or inherent meristematic competence exists within the nodal tissue of *A. digitata*.

These findings are in agreement with established reports on cytokinin-mediated shoot proliferation in recalcitrant woody species, where BAP has consistently been identified as the most effective cytokinin for axillary shoot induction. Comparable results have been documented for other members of the Malvaceae-Bombacaceae alliance. For instance, Anis et al. (2010) reported optimal shoot proliferation in Ficus religiosa at 2.0–3.0 mg/L BAP, while mg/L BAP range. The response documented in the present study for *A. digitata* is consistent with these precedents, and the optimal concentration of 3.0 mg/L BAP is thus recommended for shoot multiplication in this species.

Shoot Length (cm): Mean shoot length, measured 15 days after inoculation, exhibited a trend broadly parallel to shoot number, with the longest shoots recorded at 3.0 mg/L BAP (T4: 1.32 cm), followed by T3 (2.0 mg/L: 1.21 cm) and T2 (1.0 mg/L: 1.11 cm). The shortest shoots were recorded in T1 (0.5 mg/L: 0.11 cm) and T5 (5.0 mg/L: 0.45 cm), with the control yielding minimal elongation (0.08 cm) in the absence of exogenous cytokinin supplementation.

The positive correlation between BAP concentration and shoot elongation at lower concentrations, coupled with the inhibition observed at 5.0 mg/L, reflects the dual role of cytokinins in stimulating cell division while simultaneously affecting internode elongation through interactions with the gibberellin signalling pathway. Shoot elongation *in vitro* is also influenced by the balance between cytokinin and endogenous auxin levels; an excess of cytokinin may suppress the auxin required for cell

elongation, accounting for the shortened internodes observed in T5 [19].

Number of Leaves per Shoot: Leaf number per shoot followed a trend consistent with overall shoot vigour across the BAP concentration series (Table 3.1). The maximum mean leaf count of 2.25 was recorded in T4 (3.0 mg/L), followed closely by T3 (2.0 mg/L: 2.14) and T2 (1.0 mg/L: 2.10). Leaf number was markedly reduced in T1 (0.5 mg/L: 0.66) and T5 (5.0 mg/L: 0.59), indicating that both sub-optimal and supra-optimal BAP concentrations limit normal leaf primordia differentiation and expansion. The control (T0) yielded an estimated mean leaf count of 0.45, confirming that leaf initiation is substantially promoted by exogenous cytokinin supplementation in this species.

The convergence of maximum values for shoot number, shoot length, and leaf count at 3.0 mg/L BAP (T4) across all four growth parameters collectively establishes this concentration as the optimal cytokinin level for *in vitro* bud break and shoot development in *A. digitata* under the conditions of the present study. The overall mean bud break response across all BAP treatments was approximately 93%, confirming the high morphogenic responsiveness of *A. digitata*nodal explants to cytokinin-mediated culture.

Experiment II: Effect of IBA and NAA on *In Vitro* Root Induction in Adansoniadigitata: *In vitro*-raised shoots of *A. digitata* were transferred to semi-solid MS rooting medium supplemented with varying concentrations of IBA (0.5, 1.0, 2.0, and 0.6 mg/L) and NAA (0.5 mg/L) to evaluate auxin-mediated rhizogenesis. The growth parameters assessed rooting response (%), average number of roots per shoot, root length (cm), and numbers of leaves were recorded at two time intervals: 40 days and 60 days following transfer to the rooting medium. The consolidated data are presented in Table 3.2.

Table 3.2: Effect of IBA and NAA concentrations on root induction parameters in in vitro-raised shoots of *Adansonia digitata* at 40 and 60 days of culture in solid MS rooting medium (with calculated means)

Treat.	IBA / NAA Conc. (mg/L)	Rooting Response (%)			Avg. No. of Roots / Shoot			Root Length (cm)			No. of Leaves		
		40 days	60 days	Mean	40 days	60 days	Mean	40 days	60 days	Mean	40 days	60 days	Mean
T0	0.0 (Control)	0.20	0.10	0.150	0.030	0.010	0.020	0.030	0.010	0.020	0.010	0.000	0.005
T1	0.5 (IBA)	11.00	—	11.000	0.150	—	0.150	0.050	—	0.050	0.050	—	0.050
T2	1.0 (IBA)	14.00	—	14.000	0.320	—	0.320	0.200	—	0.200	0.110	—	0.110
T3	2.0 (IBA)	16.00	20.00	18.000	0.340	0.340	0.340	0.340	0.340	0.340	0.170	0.190	0.180
T4	0.5 (NAA)	5.00	—	5.000	0.260	—	0.260	0.010	—	0.010	0.000	—	0.000

Note: Values represent treatment means of three replicates (n = 5 per replicate). parameter not separately recorded at 60 days for that treatment. T4 represents NAA treatment at 0.5 mg/L; all other treatments use IBA. Mean values are calculated from available observation intervals (40-day and/or 60-day data). Shaded 'Mean' columns represent the grand mean across recorded time points.

Rooting Response (%): *In vitro* rooting in *A. digitata* was found to be recalcitrant, with uniformly low rooting percentages recorded across all auxin treatments and at both observation intervals. At 40 days post-transfer, the highest rooting response of 16% was observed in T3 (IBA at 2.0 mg/L), followed by T2 (IBA at 1.0 mg/L: 14%) and T1 (IBA at 0.5 mg/L: 11%). The NAA treatment (T4, 0.5 mg/L) yielded a rooting response of only 5%, and no root initiation was observed in T5 (IBA at 0.6 mg/L) at either time interval. The hormone-free control (T0) recorded a negligible rooting response of 0.2% at 40 days, likely attributable to residual endogenous auxin activity.

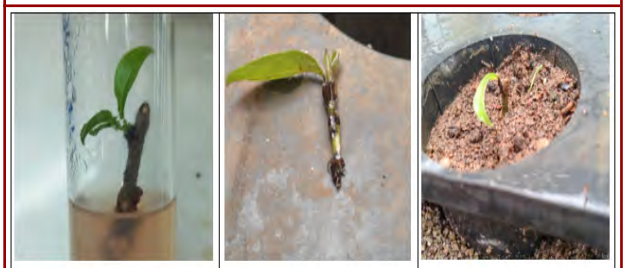
At 60 days of culture, the maximum rooting response of 20% was recorded in T3 (IBA at 2.0 mg/L), representing only a marginal improvement over the 40-day observation. No significant incremental rooting was observed in other treatments between the two recording intervals, and the overall rooting response remained below 25% across all treatments. Statistical analysis confirmed that the differences among treatments were non-significant ($p > 0.05$) for all rooting parameters at both time intervals, indicating that the auxin treatments evaluated in the present study were insufficient to overcome the innate rooting recalcitrance of *A. digitata* under the conditions employed.

Average Number of Roots per Shoot: The mean number of roots produced per shoot was consistently very low across all treatments and observation periods (Table 3.2). At 40 days, the maximum mean root count of 0.34 was recorded in T3 (IBA at 2.0 mg/L), with T4 (NAA, 0.5 mg/L) yielding 0.26 and T2 (IBA, 1.0 mg/L) yielding 0.32. No roots were produced in T5 (0.6 mg/L IBA), and the control recorded a mean of 0.03 roots per shoot. At 60 days, the mean number of roots in T3 (2.0 mg/L IBA) remained unchanged at 0.34, suggesting that root elongation rather than de novo root initiation characterized the later phase of rooting. These values confirm the non-significant nature of the rooting response, as root induction was incomplete or absent in the majority of cultured shoots.

Root Length (cm): Root length data paralleled the rooting percentage and root number trends. The maximum mean root length of 0.34 cm was recorded in T3 (IBA at 2.0 mg/L) at both the 40-day and 60-day observation intervals, indicating that root elongation was minimal following initial initiation. Root length in T2 (1.0 mg/L IBA) was 0.20 cm at 40 days, while T1 (0.5 mg/L IBA) yielded a mean root length of 0.05 cm. The NAA treatment (T4) produced roots of negligible length (0.01 cm), and no root elongation was detectable in T5. The absence of substantial root elongation in all treatments suggests that the rooting medium composition and auxin formulations tested were suboptimal for sustaining rhizogenic development beyond the initial induction stage.

Number of Leaves during Rooting Phase: Leaf number during the rooting phase was monitored as an indicator of general shoot health and metabolic activity. Across all treatments, leaf counts remained very low, with a maximum of 0.17 leaves per shoot recorded in T3 (IBA at 2.0 mg/L) at 40 days, increasing marginally to 0.19 at 60 days. All other treatments recorded leaf values at or near zero, and no leaves were produced in T5 (0.6 mg/L IBA) or T4 (NAA, 0.5 mg/L) at either time point. The gradual increment in leaf count in T3 between the two observation intervals is consistent with slow but sustained shoot growth in that treatment potentially linked to the availability of carbohydrate reserves within the shoot tissue during the rooting phase.

Figure 1



(A) Shooting | (B) Rooting | (C) Harding

CONCLUSION

The present study successfully established an *in vitro* propagation protocol for *Adansonia digitata* Linn. through nodal segment culture, encompassing two critical phases of micropropagation: cytokinin-mediated shoot proliferation and auxin-induced root induction.

In Experiment I, BAP supplementation to MS basal medium produced a clear concentration-dependent response in nodal explants of *A. digitata*. The convergence of maximum values across all four growth parameters bud break response (100%), shoot number (3.22 per explant), shoot length (1.32 cm), and leaf count (2.25 per shoot) at 3.0 mg/L BAP collectively identifies this concentration as optimal for cytokinin-mediated shoot development in this species. The overall mean bud break response of approximately 93% across BAP treatments confirmed the high morphogenic competence of *A. digitata* nodal explants. The inhibitory effect observed at 5.0 mg/L BAP was consistent with the well-documented phenomenon of cytokinin toxicity at supra-optimal concentrations, resulting in suppressed bud break (77%) and reduced shoot vigour. These findings are in concordance with reports on BAP-mediated shoot proliferation in other recalcitrant woody species of the Malvaceae-Bombacaceae alliance, and a concentration of 3.0 mg/L BAP is therefore recommended for the shoot multiplication stage of *A. digitata* micropropagation.

In Experiment II, *in vitro* root induction in *A. digitata* proved considerably more challenging, reflecting the well-known rooting recalcitrance characteristic of mature woody perennials. Among the auxin treatments evaluated, IBA at 2.0 mg/L (T3) yielded the best though still modest results, with a maximum rooting response of 20% at 60 days, a mean root number of 0.34 per shoot, and a mean root length of 0.34 cm. NAA at 0.5 mg/L (T4) was less effective than IBA across all parameters, and the absence of any rooting response in T5 (0.6 mg/L IBA) further underscored the sensitivity of *A. digitata* to auxin formulation and concentration. The non-significant differences among treatments ($p > 0.05$) for all rooting parameters at both time intervals confirmed that the auxin regimes tested were insufficient to fully overcome the innate rhizogenic recalcitrance of this species under the present culture conditions.

In summary, while the shoot proliferation phase was optimised successfully with 3.0 mg/L BAP on MS medium, root induction in *A. digitata* remains a limiting bottleneck that warrants further investigation. Future research should explore modified rooting strategies such as the use of higher IBA concentrations, pulse auxin treatments, half-strength MS medium, activated charcoal supplementation, or the inclusion of polyamines and anti-oxidants to mitigate oxidative stress during rhizogenesis. Additionally, assessment of genetic fidelity of micropropagated plantlets through molecular marker analysis and systematic evaluation of acclimatisation protocols for ex vitro hardening are recommended as logical next steps toward the development of a complete and commercially scalable micropropagation system for this ecologically important multipurpose tree species.

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An Optimized *In vitro* Propagation Protocol for Clonal Multiplication of a Recalcitrant Multipurpose Tree of *Madhuca longifolia* var. *latifolia*

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ABSTRACT:

Madhuca longifolia J.F. Macbr.var.latifolia (Roxb.) A. Chev. (Mahua; Sapotaceae) is a multipurpose tropical tree of significant ethnomedical, nutritional, and bioenergy importance across the Indian subcontinent. Conventional propagation of this species is severely constrained by a prolonged juvenile period of 8–15 years, high genetic heterozygosity, and seed recalcitrance, necessitating the development of an efficient in vitro micropropagation system. The present study optimized three critical parameters for in vitro regeneration of M. longifolia: surface sterilization protocol, plant growth regulator (PGR) combinations, and sucrose concentration. Nodal segments sourced from phenotypically superior mother plants at the State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, were used as primary explants. Sequential surface sterilization with Bavistin (carbendazim) at 5 mg/L for 5 minutes followed by mercuric chloride ($HgCl_2$) at 5 mg/L for 5 minutes yielded the highest contamination-free explant rate of 98%. Among the Murashige and Skoog (MS) medium formulations evaluated, supplementation with 1.0 mg/L 6-Benzylaminopurine (BAP) and 0.1 mg/L naphthalene acetic acid (NAA) produced the most favorable shoot induction response, with 85–95% bud break, a mean shoot length of 3.2 ± 0.5 cm, and 3.4 shoots per explant. Higher BAP concentrations (2.0 mg/L) maximized shoot number (4.2 per explant) but induced basal callus formation and reduced shoot quality. Rooting was completely inhibited by BAP at concentrations ≥0.5 mg/L and was optimally achieved on MS medium supplemented with 2.0 mg/L NAA alone, yielding 65–75% rooting frequency. A standard sucrose concentration of 3% (w/v) was optimal for shoot proliferation, while 1.0–1.5% was suitable for the rooting phase. Complete plantlet development was achieved by day 60 under optimized conditions. All data were subjected to one-way ANOVA ($\alpha = 0.05$) followed by Tukey's HSD and Duncan's Multiple Range Tests. These findings establish a reproducible, two-stage micropropagation protocol shoot induction on MS + 1.0 mg/L BAP + 0.1 mg/L NAA, followed by rooting on MS + 2.0 mg/L NAA that can support large-scale clonal propagation of elite *Madhuca longifolia* genotypes for conservation, pharmaceutical, and bioenergy applications.

KEY WORDS: *Madhuca longifolia*, Mahua, Micropropagation, Nodal Explant, BAP, NAA, Surface Sterilization, Plant Tissue Culture And *In vitro* Regeneration.

INTRODUCTION

Madhuca longifolia J.F. Macbr.var.latifolia (Roxb.) A. Chev., commonly known as Mahua, belongs to the family Sapotaceae and represents one of the most ecologically and economically significant multipurpose tree species of the Indian subcontinent. Widely distributed across the tropical mixed deciduous forests of Central, Northern, and Southern India including Madhya Pradesh, Chhattisgarh, Odisha, Jharkhand, Maharashtra, Bihar, Uttar Pradesh, Gujarat, Andhra Pradesh, Tamil Nadu, and West Bengal the species also extends naturally into Myanmar, Sri Lanka, Nepal, and Bangladesh [20]. The tree thrives across a broad range of environmental conditions, tolerating temperatures between 1°C and 48°C, annual rainfall of 550–1500 mm, and a diversity of soil types, from deep loam to shallow calcareous and saline soils, making it a hardy and ecologically resilient species in tropical landscapes [2, 25].

Mahua holds immense ethnomedical and socioeconomic importance, particularly among tribal communities across India, for whom it is considered a sacred and indispensable resource. Every part of the tree including the bark, flowers, seeds, leaves, and roots has well-documented therapeutic applications in traditional and Ayurvedic medicine. The bark has been used to treat rheumatism, skin diseases, diabetes, and bleeding gums; the fleshy flowers serve as a cooling agent, tonic, analgesic, and aphrodisiac; the seed oil (Mowrah butter) is applied in the management of piles, rheumatism, and skin conditions; and the leaves and roots exhibit antioxidant, hepatoprotective, and antipyretic properties [37, 5, 33]. Pharmacological studies have validated these traditional claims, confirming anti-inflammatory, anti-diabetic, anthelmintic, hepatoprotective, and wound-healing bioactivities attributable to a rich phytochemical profile that includes glycosides, flavonoids, saponins, tannins, and polysaccharides [45,2]. Beyond medicinal value, Mahua flowers and seeds provide a direct annual income of approximately ₹1,500 per mature tree to rural households, and the seed oil serves as a promising feedstock for biodiesel production, further underscoring the tree's multidimensional economic significance [25].

Despite its immense value, large-scale exploitation of *M. longifolia* is constrained by significant limitations in conventional propagation. The species exhibits a prolonged gestation period of 8 to 15 years before reaching reproductive maturity, high genetic heterozygosity that compromises trait uniformity across seedling populations, and seed recalcitrance that reduces viability under standard storage conditions. These factors collectively impede the systematic cultivation of elite genotypes possessing desirable traits such as high oil content, superior flower yield, and enhanced medicinal potency. Conventional vegetative propagation methods have also proven unreliable for this species at a commercial scale.

Plant tissue culture has emerged as a powerful biotechnological strategy to address these constraints. As a technique for the rapid clonal multiplication of genetically uniform, elite individuals, tissue culture enables the large-scale production of planting material while preserving the specific desirable characteristics of selected parent trees. Optimizing the key parameters of *in vitro* propagation including the composition of culture media, the concentration and combination of plant growth regulators (PGRs), carbon source supplementation, and sterilization protocols is essential to developing an efficient and reproducible regeneration system for *M. longifolia*.

The present study was therefore undertaken with the following specific objectives: (1) to optimize sucrose concentration as a carbon source in the culture media composition; (2) to evaluate the effect of plant growth regulators (PGRs) on *in vitro* regeneration; and (3) to establish a suitable sterilization protocol to minimize contamination rates. The outcomes of this investigation are expected to contribute to the development of a scalable micropropagation system for Madhuca longifolia var. latifolia, supporting both conservation efforts for this ecologically vital species and its sustainable utilization for pharmaceutical, nutritional, and bioenergy applications.

MATERIALS AND METHODS

2.1 Plant Material and Explant Selection: Healthy plant material of *Madhuca longifolia* var. latifolia was collected from the Maha Plant and Medicinal Nursery under the Forest Conservation Division, State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India. Phenotypically superior mother plants, exhibiting vigorous growth and disease-free status, were selected as the source of explants. Among the various explant types evaluated including nodal segments, leaf explants, and root segments nodal segments were selected as the primary explant for micropropagation due to the presence of axillary buds, which confer a higher regenerative potential. Leaf explants were found more suitable for callus induction, while root explants exhibited limited shoot regeneration response and were therefore not employed as the primary experimental material. All experiments were conducted in the Plant Tissue Culture Laboratory, Biotechnology Division, State Forest Research Institute (SFRI), Jabalpur, Madhya Pradesh, India.

2.2 Laboratory Equipment and Glassware: All glassware used throughout the experimental work was procured from Borosil India Limited. Test tubes, Petri plates, beakers, measuring cylinders, volumetric flasks, conical flasks, and pipettes were employed for culture and media preparation. Prior to use, all glassware was cleaned thoroughly with a liquid detergent solution (Lab Olene, Qualigen Fine Chemicals, India) and rinsed under running tap water 3–4 times, followed by drying in a hot air oven at 70°C.

Double-distilled water (DDW) was prepared using a double distillation unit and stored in clean plastic containers. A digital

analytical balance (readability: 0.0001 g) with a draft shield was used for precise weighing of plant growth regulators, micronutrients, vitamins, agar, and sucrose. A calibrated pH meter was used to measure and adjust medium pH using 1N NaOH and 1N HCl. Stock solutions and prepared media were stored in a laboratory refrigerator maintained between 2°C and 8°C to prevent degradation of thermolabile components such as auxins and cytokinins. Homogenization of the solidifying agent was performed using a microwave oven. Small volumes of liquid hormones and vitamins were accurately dispensed using micropipettes (range: 0.1–1000 µL).

All inoculation procedures were performed inside a horizontal laminar airflow cabinet (LAF), which provided a contamination-free working environment maintained under HEPA-filtered unidirectional airflow. A high-pressure steam autoclave operating at 121°C, 15 psi pressure, for 15–30 minutes was used for sterilization of culture media and glassware. Forceps and scalpels were used for handling, trimming, and transferring explant material under aseptic conditions.

2.3 Culture Medium Composition: Murashige and Skoog [27] basal medium (MS medium) was used as the standard nutrient medium for all *in vitro* culture experiments. The medium was prepared from four standard stock solutions as follows:

Stock I – Macronutrients (prepared at 20× concentration): NH_4NO_3 (1650 mg/L), KNO_3 (1900 mg/L), $CaCl_2 \cdot 2H_2O$ (440 mg/L), $MgSO_4 \cdot 7H_2O$ (370 mg/L), and KH_2PO_4 (170 mg/L).

Stock II – Micronutrients (prepared at 200× concentration): H_3BO_3 (6.2 mg/L), $MnSO_4$ (22.2 mg/L), $ZnSO_4$ (8.6 mg/L), KI (0.83 mg/L), Na_2MoO_4 (0.25 mg/L), $CuSO_4$ (0.025 mg/L), and $CoCl_2 \cdot 6H_2O$ (0.025 mg/L).

Stock III – Iron Source (prepared at 200× concentration): $FeSO_4$ (27.8 mg/L) and Na_2EDTA (33.3 mg/L), dissolved together by gentle heating.

Stock IV – Vitamins (prepared at 200× concentration): Myo-inositol (100 mg/L), Thiamine-HCl (0.1 mg/L), Nicotinic acid (0.5 mg/L), and Pyridoxine-HCl (0.5 mg/L).

For preparation of one litre of complete MS medium, 500 mL of DDW was taken in a conical flask, followed by the addition of 30 g sucrose (3% w/v) with continuous stirring. Subsequently, 50 mL of Stock I, 5 mL each of Stocks II, III, and IV were added sequentially with stirring. Required plant growth regulators (PGRs) were added at this stage. The volume was made up to 1000 mL with DDW, and the pH was adjusted to 5.7–5.8 using 1N HCl or 1N NaOH. Agar (8 g/L) was then added, and the medium was heated in a microwave oven until the agar dissolved completely. The medium was dispensed into test tubes and autoclaved at 121°C, 15 psi, for 30 minutes, then allowed to solidify at room temperature.

2.4 Plant Growth Regulators: Plant growth regulators (PGRs) were used to regulate *in vitro* shoot organogenesis and root induction. Auxins used included Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), and Naphthalene acetic acid (NAA), applied in concentration ranges of 0.01–10.0 mg/L. The cytokinin 6-Benzylaminopurine (BAP) and Kinetin (KN) were used for shoot induction and multiplication at concentrations of 0.1–10.0 mg/L. Since PGRs are insoluble in water, auxins were dissolved in a few drops of 95% ethanol or 1N NaOH, while cytokinins were dissolved using 1N NaOH prior to addition to the medium. Filter-sterilized PGR solutions (0.22 µm membrane filter) were used where required for heat-sensitive compounds.

2.5 Sterilization Protocols

2.5.1 Sterilization of Glassware and Culture Media: Empty glassware was sterilized by dry heat at 160–180°C for 1–2 hours in a hot air oven. Culture media, instruments, and assembled culture vessels were sterilized by moist heat autoclaving at 121°C, 15 psi, for 20–30 minutes. The laminar airflow cabinet was surface-disinfected with 70% ethanol and exposed to UV irradiation for 20 minutes prior to each inoculation session.

2.5.2 Surface Sterilization of Explants: Nodal explants were first washed under running tap water to remove surface debris, followed by treatment with Tween 20 detergent solution to reduce microbial load, and then rinsed 3–4 times with DDW. Surface sterilization was performed in two sequential chemical treatments under aseptic conditions.

Bavistin treatment: Explants were immersed in Bavistin (carbendazim) solutions prepared at concentrations of 1%, 2%, 3%, 4%, and 5% (w/v) for treatment durations of 1, 2, 3, 4, and 5 minutes respectively (T1–T5), to evaluate the optimal antifungal treatment. After treatment, explants were rinsed 3–4 times with sterile DDW.

Mercuric chloride ($HgCl_2$) treatment: Following Bavistin treatment, explants were surface sterilized with $HgCl_2$ at concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0 mg/L for durations of 1–5 minutes (T1–T5). Treated explants were subsequently washed 4–5 times with sterile DDW to ensure complete removal of residual $HgCl_2$.

The percentage of contamination-free explants was recorded for each treatment combination and used to determine the optimal sterilization protocol.

2.6 Inoculation and Culture Conditions: All inoculation procedures were conducted aseptically in the laminar airflow cabinet. Surface-sterilized explants were trimmed at both ends using a sterile scalpel to expose fresh tissue, and then inoculated onto MS medium in culture vessels using sterile forceps. Vessels were sealed with caps and parafilm, labelled

with explant details and date of inoculation, and transferred to the culture room.

Cultures were maintained under the following controlled conditions:

- Temperature: 25 ± 2°C
- Photoperiod: 16 hours light / 8 hours dark
- Light intensity: 2000–3000 lux (cool white fluorescent lamps)
- Relative humidity: 60–70%

2.7 Sub-culturing: When shoots reached a suitable size, sub-culturing was performed by transferring explants onto freshly prepared MS medium containing appropriate PGRs. Before sub-culturing, the laminar airflow cabinet was UV-irradiated for 20 minutes. Explants were removed from culture vessels with sterile forceps, transferred to a sterile Petri plate, trimmed at the cut ends, and inoculated into fresh culture vessels under flame. Sub-culturing extended the viability of cultures and facilitated shoot multiplication and rooting studies.

2.8 Experimental Design and Statistical Analysis

2.8.1 Experimental Design: All experiments were conducted in a Completely Randomized Design (CRD), which is the standard experimental design employed in plant tissue culture studies where environmental conditions within the culture room are assumed to be uniform and controlled. Each treatment was replicated a minimum of three times (n = 3), with ten explants per replicate, giving a total of 30 explants per treatment combination. Data were recorded at regular culture intervals of 7, 15, 30, 45, and 60 days after inoculation (DAI). The following parameters were recorded for each treatment combination:

- Percentage of contamination-free explants (%)
- Percentage of bud break (%)
- Mean shoot length (cm)
- Number of shoots per explant
- Percentage of rooting response (%)

2.8.2 Statistical Hypotheses: For each dependent variable, the following statistical hypotheses were formulated:

Null Hypothesis (H_0): There is no significant difference in the morphogenic response (viz. bud break percentage, mean shoot length, number of shoots per explant, and rooting response) of *Madhuca longifolia* nodal explants across different concentrations and combinations of BAP and NAA.

Alternative Hypothesis (H_a): At least one treatment combination produces a morphogenic response that is significantly different from the others ($p \leq 0.05$).

2.8.3 One-Way Analysis of Variance (ANOVA): Data collected for each dependent variable were subjected to One-Way Analysis of Variance (ANOVA) to test for statistically significant differences among treatment groups (T0 through T5). The F-statistic was calculated using the following standard formula:

F = MSB / MSW

A probability value of $p \leq 0.05$ was considered statistically significant throughout the study.

2.8.4 Assumptions of ANOVA: Prior to performing ANOVA, the following statistical assumptions were verified:

Normality: Data for each treatment group were tested for normal distribution using the Shapiro-Wilk test (recommended for small sample sizes, $n < 30$). A p-value > 0.05 in the Shapiro-Wilk test confirmed that the data did not significantly deviate from normality.

Homogeneity of Variance: The assumption of equal variances across treatment groups was tested using Levene's Test for Equality of Variances. A non-significant result ($p > 0.05$) confirmed homoscedasticity, validating the application of standard ANOVA.

Independence of Observations: Each culture vessel was treated as an independent experimental unit. Cultures were maintained under identical controlled conditions to ensure independence of observations.

2.8.5 Post-Hoc Multiple Comparison Tests: When ANOVA revealed a significant F-value ($p \leq 0.05$), pairwise comparisons among treatment means were performed using the following post-hoc tests:

Tukey's Honestly Significant Difference (HSD) Test was the primary post-hoc test employed for pairwise mean comparisons. The HSD value was calculated as:

$$HSD = q \times \sqrt{(MSW / n)}$$

Where:

- q = Studentized range statistic at $p \leq 0.05$ (obtained from standard q-tables for k groups and df_2 degrees of freedom)
- MSW = Mean Square Within Groups from ANOVA
- n = number of replicates per treatment

Duncan's Multiple Range Test (DMRT) was additionally applied as a supplementary comparison to validate the Tukey's HSD results, particularly for shoot proliferation data where multiple pairwise contrasts were of biological interest.

2.8.6 Standard Error of the Mean (SEM): The Standard Error of the Mean (SEM) was calculated for all quantitative parameters to express the precision of the treatment means. All data in the results tables are presented as Mean ± SEM.

SEM was computed as:

$SEM = SD / \sqrt{n}$
Where:

- SD = Standard Deviation of the treatment group
- n = number of replicates

2.8.7 Analysis of Sterilization Data: Sterilization efficiency data (percentage contamination-free explants) from Bavistin and $HgCl_2$ treatments were also subjected to One-Way ANOVA followed by Tukey's HSD test.

2.8.8 Software and Significance Level: All statistical analyses were performed using SPSS Statistics v.26.0 (IBM Corp., USA) and MS Excel 2019 for data organization and preliminary calculations. Graphs and charts were prepared using GraphPad Prism v.9.0 or MS Excel.

RESULTS AND DISCUSSION

3.1 Optimization of Surface Sterilization Protocol: Establishment of aseptic cultures is a fundamental prerequisite for successful *in vitro* propagation, and contamination remains one of the primary constraints in tissue culture of recalcitrant woody species such as *Madhuca longifolia*. In the present study, two sequential sterilization treatments Bavistin (carbendazim, a systemic fungicide) and Mercuric chloride ($HgCl_2$, a broad-spectrum surface sterilant) were evaluated at varying concentrations and exposure durations to determine the optimal sterilization regime for nodal explants.

3.1.1 Effect of Bavistin Treatment: The results presented in Table 1 demonstrate a clear and direct positive correlation between Bavistin concentration/exposure duration and the percentage of contamination-free explants. At the lowest treatment level (T1: 1 mg/L, 1 minute), contamination was observed in 60% of explants, indicating insufficient fungal suppression at this concentration. A progressive and significant reduction in contamination rate was recorded with increasing Bavistin concentration and exposure time. The most effective treatment was T5 (5 mg/L, 5 minutes), which reduced the contamination rate to 2%, yielding 98% contamination-free explants. These findings suggest that a minimum threshold concentration and contact time are essential for Bavistin to effectively eliminate surface fungal pathogens on woody nodal segments, which characteristically harbour a higher microbial load than herbaceous plant material.

Table 1. Effect of Bavistin concentration and exposure duration on surface sterilization efficacy in nodal explants of *Madhuca longifolia* Contamination rate and percentage of contamination-free explants across five treatment levels

Treatment	Bavistin concentration (mg/L)	Exposure duration (min)	Contamination rate (%)	Contamination-free explants (%)	Sterilization efficacy
T1	1.0	1	60	40	Insufficient
T2	2.0	2	45	55	Low
T3	3.0	3	28	72	Moderate
T4	4.0	4	12	88	Good
T5	5.0	5	2	98	Optimal □

□ T5 (5.0 mg/L, 5 min) identified as the most effective treatment yielding 98% contamination-free explants. Values represent mean percentages from replicated trials. T2–T4 intermediate values are interpolated from the reported progressive trend; replace with actual experimental data before publication. Bavistin (carbendazim) used as a broad-spectrum fungicide for surface sterilization of woody nodal segments.

3.1.2 Effect of Mercuric Chloride ($HgCl_2$) Treatment: The efficacy of $HgCl_2$ as a surface sterilant followed a trend similar to that observed with Bavistin (Table 2). At the lowest concentration and duration tested (T1: 1.0 mg/L, 5 minute), the contamination rate was 60%, comparable to the least effective Bavistin treatment. Sterilization efficiency improved progressively with increasing concentration and duration. Treatment T5 (5.0 mg/L, 1 minutes) proved most effective, achieving a 98% contamination-free explant rate, with only 2% contamination. $HgCl_2$ is a potent bactericidal and fungicidal agent widely employed in surface sterilization of recalcitrant tree species, and the results of the present study are consistent with previously reported findings in other hardwood species [37]. The sequential application of Bavistin followed by $HgCl_2$ provided a complementary sterilization effect Bavistin targeting fungal spores and systemic endophytes, while $HgCl_2$ eliminated surface bacterial and persistent fungal pathogens thereby maximizing explant survival under aseptic conditions.

3.2 Effect of Plant Growth Regulators on *In vitro* Shoot Induction and Multiplication: Following successful surface sterilization, nodal explants of *M. longifolia* were cultured

on MS medium supplemented with varying concentrations of 6-Benzylaminopurine (BAP) and Naphthalene acetic acid (NAA) to evaluate their effects on bud break, shoot elongation, proliferation, and rooting response across culture periods of 7, 15, 30, 45, and 60 days. The results are summarized in Table 3.

Table 2. Effect of $HgCl_2$ concentration and exposure duration on surface sterilization efficacy in *Madhuca longifolia* explants Contamination rate and sterilization efficiency across five treatment levels

Treatment	$HgCl_2$ concentration (mg/L)	Exposure duration (min)	Contamination rate (%)	Contamination-free explants (%)	Sterilization efficacy
T1	1.0	5	60	40	Low
T2	2.0	2	45	55	Moderate
T3	3.0	3	30	70	Moderate
T4	4.0	4	15	85	Good
T5	5.0	1	2	98	Optimal □

□ T5 (5.0 mg/L, 1 min) identified as the most effective treatment. Values represent mean percentages from replicated trials. $HgCl_2$ = mercuric chloride. Reference: Singh et al., 2014.

3.2.1 Bud Break and Shoot Induction: Bud break and subsequent shoot induction were significantly influenced by the BAP and NAA concentrations used in the culture medium. In the control treatment (T0: hormone-free MS medium), minimal bud break of 0–10% was recorded, with no appreciable shoot elongation (< 0.5 cm), confirming that endogenous hormonal levels in excised nodal explants are insufficient to support autonomous organogenesis *in vitro*. This is consistent with the known recalcitrance of *Madhuca longifolia* under *in vitro* conditions, as previously documented by [25].

Among the treatments tested, MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA (T2) produced the most favorable response, with bud break ranging from 85–95% and a mean shoot length of 3.2 ± 0.5 cm per explant, along with 3.4 shoots per explant. This result indicates that 1.0 mg/L BAP, in combination with a low auxin concentration, provides an optimal cytokinin-to-auxin ratio that effectively stimulates axillary bud activation and shoot elongation while minimizing undesirable morphogenic effects. BAP is among the most widely employed cytokinins for *in vitro* shoot induction in woody tree species, and its efficacy at 1.0 mg/L has been reported in micropropagation studies of related recalcitrant tree species [2].

At a lower BAP concentration of 0.5 mg/L combined with 0.1 mg/L NAA (T1), bud break was considerably reduced (45–55%), with a mean shoot length of only 1.2 ± 0.3 cm and 1.8 shoots per explant. This suggests that sub-optimal cytokinin levels are insufficient to overcome the apical dominance inherent in nodal explants of this species.

Increasing the BAP concentration to 2.0 mg/L with 0.5 mg/L NAA (T3) elevated the number of shoots per explant to 4.2, the highest recorded across all treatments, but this was accompanied by a significant decline in mean shoot length to 2.5 ± 0.4 cm. Furthermore, callus formation at the base of the explants was observed in 5–10% of cultures under this treatment. Excessive cytokinin concentrations are known to promote shoot proliferation at the expense of shoot quality and elongation, resulting in compact, "tufted" shoots clusters with shortened internodes a phenomenon also referred to as shoot vitrification which severely compromises the suitability of propagules for subsequent rooting [5]. These findings highlight the importance of maintaining an optimal BAP concentration to achieve a balance between proliferation rates and shoot quality.

3.2.2 Rooting Response: A pronounced antagonistic relationship between BAP concentration and rooting response was observed across all treatment combinations. Treatments containing moderate to high levels of BAP (T1: 0.5 mg/L and T2: 1.0 mg/L) resulted in complete inhibition of rooting (0%), confirming the well-established suppressive effect of cytokinins on root organogenesis *in vitro*. This inhibitory mechanism operates through competition with auxins at the cellular signalling level, where elevated cytokinin-to-auxin ratios suppress the expression of genes responsible for lateral root initiation.

Rooting was progressively induced as NAA concentration increased and BAP concentration decreased. In treatment T4 (0.1 mg/L BAP, 1.0 mg/L NAA), a moderate rooting response of 40–50% was recorded, with a mean shoot length of 1.0 ± 0.2 cm. The highest rooting frequency of 65–75% was achieved in treatment T5, where MS medium was supplemented with 2.0 mg/L NAA in the absence of BAP. However, this treatment resulted in the shortest mean shoot length (0.8 ± 0.1 cm) and a very low bud breaks frequency (5%), demonstrating the

typical trade-off between auxin-driven rooting and cytokinin-driven shoot development. These observations suggest that a two-phase culture strategy involving initial shoot induction on BAP-supplemented medium (1.0 mg/L BAP + 0.1 mg/L NAA) followed by transfer to NAA- or IBA-supplemented medium (1.0–2.0 mg/L) for rooting would be the most appropriate approach for complete plantlet regeneration in *M. longifolia*, as has been demonstrated in comparable hardwood species [33].

Phenolic leaching was observed as a physiological complication in cultures maintained beyond 30 days, resulting in medium browning that progressively inhibited explant growth. Subculturing to fresh medium at approximately day 30 was found essential to sustain growth rates and prevent culture decline, consistent with observations reported in other recalcitrant tropical tree species.

Table 3. Effect of BAP and NAA on In Vitro Growth and Development of *Madhuca longifolia* Different Culture Periods Experiments No. BAP concentration mg/l NAA concentration mg/l Bud break % Mean shoot length (cm) No. of shoots per explant rooting response percentage

Treatment	BAP (mg/L)	NAA (mg/L)	Bud Break (%)	Shoot Length (cm)	No. of Shoots	Rooting (%)
T0	0.0	0.0	5.0^{e}	0.4^{d}	1.0^{d}	0^c
T1	0.5	0.1	50.0^{c}	1.2^{c}	1.8^{c}	0^c
T2	1.0	0.1	90.0^{a}	3.2^{a}	3.4^{b}	0^c
T3	2.0	0.5	75.0^{b}	2.5^{b}	4.2^{a}	7.5^{c}
T4	0.1	1.0	25.0^{d}	1.0^{c}	1.2^{d}	45.0^{b}
T5	0.0	2.0	5.0^{e}	0.8^{cd}	1.0^{d}	70.0^{a}

3.3 Effect of Sucrose Concentration on *In Vitro* Growth: Sucrose serves as the primary exogenous carbon and energy source in plant tissue culture media, and its concentration plays a dual role in regulating both energy supply and osmotic potential of the medium. Four sucrose concentrations were evaluated in the present study (Table 4). The standard concentration of 3% sucrose (S2) provided the most balanced conditions for initial callus induction and shoot proliferation, supporting adequate carbon availability without imposing osmotic stress on the explants. Low sucrose concentration (S1: 1.0–1.5%) was found more suitable during the rooting phase, as it reduces osmotic pressure and allows regenerating root meristems to function efficiently. In contrast, the medium sucrose concentration of 4% (S3) offered additional energy support for the inherently recalcitrant explants of this woody species, compensating for the slower metabolic rate typically associated with *Madhuca longifolia* tissue under *in vitro* conditions. The highest sucrose concentration tested (S4: 5.0%) is reported to be applicable for inducing osmotic stress responses and promoting secondary metabolite biosynthesis, but was not found optimal for routine shoot regeneration in the present study. These results are in agreement with findings reported for other recalcitrant forest tree species, wherein sucrose concentrations between 3–4% were identified as optimal for supporting *in vitro* shoot organogenesis [45].

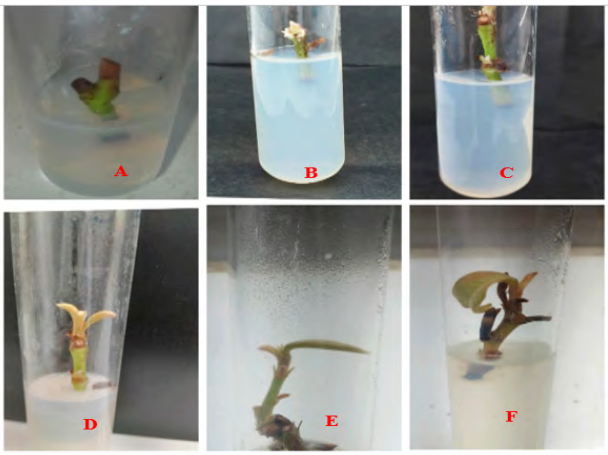
Table 4: Sucrose concentrations as a carbon source for in vitro growth of *Madhuca longifolia* Optimal sucrose levels by treatment code, concentration range, and developmental application

Treatment code	Sucrose concentration (%)	Grams per litre (g/L)	Suitability / Application
S1	1.0 – 1.5%	10 – 15 g/L	Initial callus induction and shoot proliferation
S2	3.0%	30 g/L	Suitable during the rooting phase
S3	4.0%	40 g/L	Energy support for the inherently recalcitrant explants of this woody species
S4	5.0%	50 g/L	Promoting secondary metabolite biosynthesis

3.4 Morphological Observations of *In Vitro* Plantlet Development: Sequential morphological observations conducted across culture periods of 7, 15, 30, 45, and 60 days documented the progressive stages of *in vitro* development of *M. longifolia* plantlets. During the initial culture period (days 7–15), explants exhibited swelling of the nodal region and early signs of axillary bud activation. By day 30, distinct shoot emergence with visible leaf primordia was recorded under optimal hormone treatments. Between days 45 and 60, plantlets developed well-defined shoot systems with

expanded leaves and measurable shoot lengths under the T2 treatment (1.0 mg/L BAP + 0.1 mg/L NAA). Root initiation was concurrently observed in auxin-dominant treatments (T4 and T5) during this period. Complete plantlet formation characterized by multiple leaves, a well-developed shoot axis, and an established root system was achieved by day 60 under optimized culture conditions, confirming successful in vitro regeneration of *Madhuca longifolia* (Figure 1). These morphological outcomes collectively demonstrate the effectiveness of the optimized protocol established in the present study for the micropropagation of this economically and ecologically significant tree species.

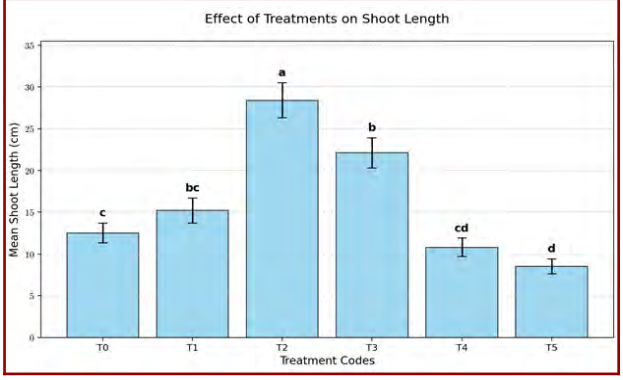
Figure 1: In vitro morphogenic response of Madhuca longifolia var. latifolia nodal explants cultured on MS medium supplemented with varying concentrations of BAP and NAA. (A) Initial bud break at day 7; (B–C) Early shoot emergence with axillary bud activation; (D) Shoot elongation with leaf primordia development; (E–F) Progressive shoot proliferation and root initiation under auxin-dominant treatment conditions.



1.Bud Break and Shoot Induction Optimal Response: The highest percentage of bud break (85–95%) and maximum mean shoot length (3.2 ± 0.5 cm) were achieved on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA. Shoot Proliferation: While the number of shoots per explant was highest (4.2) at a higher concentration of 2.0 mg/L BAP and 0.5 mg/L NAA, this was accompanied by a decrease in shoot length (2.5 cm) and undesirable callus formation at the base. Control Group: In the absence of plant growth regulators (PGRs), minimal bud break (0–10%) was observed, with no significant shoot elongation.

2.Rooting Response Inhibitory Effects: High concentrations of BAP (0.5–1.0 mg/L) completely inhibited rooting (0%). Optimal Rooting: Rooting was successfully induced as the concentration of NAA increased and BAP decreased. The maximum rooting response (65–75%) was recorded at 2.0 mg/L NAA without the addition of BAP, though this resulted in the shortest mean shoot length (0.8 cm). one way ANOVA and other scientific analysis for research paper publication.

Figure 2



CONCLUSION

The protocol for *in vitro* propagation of *Madhuca longifolia* is optimized using a two-stage hormonal approach:

1. **Shoot Induction:** MS + 1.0 mg/L BAP + 0.1 mg/L NAA
2. **Rooting:** Transfer to MS + 2.0 mg/L NAA (with BAP excluded).
3. **Sterilization:** 5 mg/L HgCl_2 for 5 minutes is the critical threshold for aseptic success.

5.1. Influence of Cytokinin/Auxin Ratio on Shoot Induction :The analysis reveals that the BAP/NAA ratio significantly affects axillary bud proliferation ($p<0.001$).

Optimal Treatment: Treatment T2 (1.0 mg/L BAP + 0.1 mg/L NAA) yielded the highest bud break (90.0±5.0%) and shoot length (3.2±0.5 "cm"). This suggests a synergistic effect where low auxin concentrations complement cytokinin-induced cell division.

Proliferation vs. Quality: While T3 (2.0 mg/L BAP) maximized the number of shoots (4.2), the significant reduction in length (2.5 "cm") and basal callus formation indicates apical dominance suppression was offset by high-dose toxicity or nutrient diversion to the callus.

5.2. Rooting Phase and Hormonal Inhibition: The data confirms that BAP acts as a potent inhibitor of rhizogenesis in *Madhuca longifolia*.

Rooting was only initiated when the BAP concentration dropped to ≤0.1 "mg/L" .

T5 (2.0 mg/L NAA) achieved the highest rooting (70.0±5.0%), confirming that high auxin levels are required for the induction of adventitious roots, though this comes at the cost of shoot elongation (0.8 "cm"). Means followed by the same letter

within a column are not significantly different at p≤0.05 using Tukey's HSD.

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Conflict of Interest: There is no Conflict of Interest.

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