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SHIVAJI UNIVERSITY, KOLHAPUR

JOURNAL OF SHIVAJI UNIVERSITY : SCIENCE AND TECHNOLOGY

(Peer Reviewed Journal)

Volume-46, Issue-2 (July, 2020)

ISSN Science -0250-5347

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ISSN Science -0250-5347

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Shivaji University, Kolhapur

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- Editor -
Prof. (Dr.) S. D. Delekar
Journal of Shivaji University :
Science and Technology

- Published by -
Dr. V. N. Shinde
Ag. Registrar,
Shivaji University, Kolhapur 416004.

- Printed by -
Shri. B. P. Patil
Superintend
Shivaji University, Kolhapur 416004.

The Editor, on behalf of the Editorial Board of the Journal of Shivaji University : Science and Technology, Vol. No. 46 (2) whises to express his thanks to the contributing authors and the experts for acting as referees for the papers included in the issue.

Published : July, 2023

Journal of Shivaji University: Science and Technology
Volume-46, Issue-2 (July, 2020)

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Department of Biotechnology

Shivaji University, Kolhapur

Journal of Shivaji University : Science & Technology

Volume-46, Issue-2 (July, 2020)

Designing Yeast: Opportunities for Synthetic Biology in Winemaking

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ABSTRACT

Synthetic biology has opened up new opportunities as a novel bioengineering technology platform to create new biological avenues for the production of bulk chemicals for industry, therapeutic drugs, and fuels. Among the various living organisms, yeast has been extensively exploited for synthetic biology research since yeast is very resourceful and has an immense commercial significance which is ideally suited for synthetic biology manipulations. Yeast is a major component in wine preparations controlling several desired properties of wine. The yeast genome has been sequenced and in conjunction with knowledge from systems biology, will be useful for designing rational yeast metabolic engineering, identification of target genes, and regulation of network balancing and expression levels of related genes. In this connection, synthetic biology methods will accelerate the precise assembly of various genes as well as their expressions and identify metabolite intermediates for improving commercial wine fermentation. The present mini-review is focused on the progress and challenges in yeast synthetic biology because of the potential opportunities in yeast designing yeast system for making better quality wines.

KEYWORDS

Biosynthetic pathways, Genome engineering, Saccharomyces cerevisiae, Synthetic biology, Wine.

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1. INTRODUCTION

The baker's yeast, *Saccharomyces cerevisiae*, is one of the earliest microorganisms to be domesticated for the production of alcoholic beverages such as wine, beer, and sake as well as for baking purposes [1-2]. Research in yeast dates back to several thousand years when their fermentative potential

was first discovered and exploited for wine production [2]. Yeast is a single-celled eukaryote fungus and free living in nature and has been domesticated for wine, bread, beer, alcohol, and proteins. Yeast cells are about 5 micrometers long and can be easily cultivated on nutrient agar plates. It divides by budding and takes 90 min for the next generation. Yeast genetics has been established since the 1950s and genetic maps with more than 1,000 loci are known. It is a model organism and is the first eukaryotic organism of which genome has been sequenced and further research is extended to microarray, hybrid protein interaction, genome-wide collections and bioinformatics analysis which has generated extensive data. Numerous studies have been carried out on transcriptomics, genomics and metabolomics enables researchers to elucidate metabolic pathways. Gene cloning in yeast consisted of *E.coli* shuttle vectors, single copy CEN vectors versus multi-copy micron vectors, selection markers for plasmids, regulatable promoters and yeast can be maintained in multiple different plasmids and has the merit of episomal plasmid versus genome integration. Yeast is the focal organism for metabolic engineering and has been designed to synthesize plant metabolites. Yeast has suitable promoters for heterologous expression and is a suitable source for relevant enzymes, codon optimization, plasmids with suitable selection markers, co-transformation, multiple expression constructs, altering expression of genes using yeast regulators/promoters, and combining different engineering steps using repeated transformation and re-usable markers plus genetic crossing.

Against this backdrop, *S. cerevisiae* is a versatile system for Synthetic Biology since its genetics and molecular cell biology are well developed. Advanced genetics and engineering tools allow goal oriented synthetic biology projects to be performed easily involving the redesign of cellular pathways and heterologous introduction and have direct biotechnological uses and a long-standing experience in the industry.

Selection of yeast strains or species for winemaking was dependent on historical and traditional knowledge rather than scientific platforms. Due to the progress in the research, newer strategies mainly mutagenesis, protoplast fusion, and breeding were employed for the yeast strain improvement for quality wine production. Synthetic biology is one step forward for refining the existing technologies for accurate and dependable results which will benefit the wine industry tremendously [3-4]. Over the last three decades, considerable knowledge has been generated concerning the physiology, biochemistry, and genetics of yeast, which has led to yeast as the primary model eukaryote in the field of genetics and molecular biology (Fig. 1).

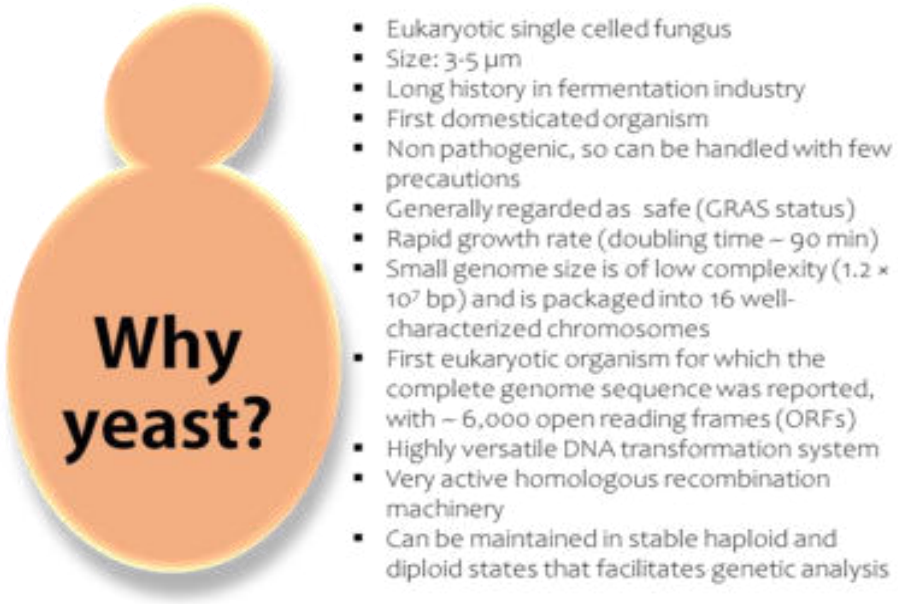

Figure 1: The key features to consider yeast as a flexible programmable biological chassis.

The wealth of information accumulated on the cellular components and their structural components through 'omics' technology has greatly facilitated the progress of yeast metabolic engineering for tailoring the yeast for the production of useful products [5-6]. Several aspects of *S. cerevisiae* fundamental metabolism have been modified and improved to meet the end bioprocess objectives in winemaking. Furthermore, the integration of data produced from different 'omics' platforms headed toward the yeast system biology provides the basis for synthetic biology and offers improved novel yeast strains for better wine production.

This review summarizes a concise overview of recent advances mainly in synthetic biology in yeast that are expected to drive this field forward rapidly for high-quality wine production. Synthetic biology is an emerging technology that merges science and engineering approaches for developing and designing novel biological functions [7]. The major thrust of synthetic biology is to construct new biological parts, devices, and systems and simultaneously change existing biological systems mainly for desirable functions. The major advantages of synthetic biology are dependable off-the-shelf parts and devices provided with standard connections, a versatile framework of yeast or bacteria that easily admit those parts, and devices that can assemble various parts into sophisticated and functional systems. The desirable results of the design and construction of the synthetic gene network established that engineering-based methodology could indeed be applied to build sophisticated, computing-like performance into biological systems. In

the framework provided by synthetic systems, biological circuits can be built from smaller well-defined parts according to model blueprints, which can be studied and tested in isolation, and their activities can be evaluated against model predictions of the system dynamics. This line of attack has subsequently been applied in the synthetic construction of additional genetic switches, memory elements, and oscillators as well as of other electronics-inspired genetic devices, including pulse generators, digital logic gates, filters, and communication modules [8]. Recently several reports have been published and elegantly reviewed by Dai et al. [9] on the integration of synthetic biology ith yeast genetic engineerings such as the rapid assembly of biosynthetic pathways [10] modulation of expression of heterologous genes [11] and localization of enzymes to a special subcellular region or scaffold [12-13]. Moreover, *S. cerevisiae* can provide a similar physical and physiological environment for the functional expression of diverse heterologous enzymes [e.g. cytochrome P450s and uridine diphosphate glycosyltransferases (UGTs)] from plants and mammals, as they allow endomembrane localization and post-translational modifications consisting mainly protein glycosylation [14-15].

2. ADVANCES IN YEAST GENOME ENGINEERING

Moving forward key concepts in yeast genetic engineering comprise the regularity of biological parts and ideas to let using those parts in gradually more intricate synthetic systems. The accomplishment of this is significantly assisted by basic technologies of DNA sequencing, cloning, and recombination. Synthetic biology, like all engineering disciplines, relies on the availability of powerful standardized tools for synthesizing, assembling, engineering, and transplanting whole genomes [16]. Yeast has also been developed as a host for receiving and propagating complete non-synthetic genomes. One of the first steps in engineering yeast genomes is to design and construct one or multiple sequence-specific endonucleases that creates DNA double-strand breaks (DSBs) at a locus of interest. The introduction of site-specific DSBs into the chromosome can greatly enhance the efficiency of introducing targeted genome modifications due to the activation of the DNA repair machinery. Many of the required tools, for example, genome synthesis, assembly, and editing methods [17-18] are broadly generic.

2.1. Chromosome Engineering

Yeast artificial chromosomes (YACs) are genetically engineered chromosomes derived from the DNA of the yeast, and then ligated into a bacterial plasmid. YACs are an ancient tool used for engineering yeast cells on the chromosomal level [19]. The YAC expression system has advantages over plasmid-based and viral gene delivery systems for introducing genes of interest into cells because these systems are limited in the size of DNA sequences they can deliver. For delivery of multiple genes, open reading frames (ORFs) are desired, since plasmid or viral vectors may be inadequate. YACs were also successfully applied for optimizing metabolic pathways

using the random assembly of pathway genes and connected promoters [20]. Moreover, recent approaches focus more on the redesign and synthesis of whole chromosomes and genomes. Recently, the total synthesis of a functional designer eukaryotic chromosome of *S. cerevisiae* was achieved by a utility of an entirely new inducible evolution system called SCRaMbLE (Synthetic Chromosome Rearrangement and Modification by LoxPsym-mediated Evolution) [21].

The SCRaMbLE system uses an inducible Cre recombinase to shuffle-up regions of the genome where loxPsym sites have been introduced. The incorporation of loxPsym sequences at sites downstream of every non-essential gene in synthetic yeast chromosome allows for Cre-mediated recombination of the genomic DNA. Cre recombinase is provided on a plasmid and its expression is induced by estradiol. It catalyzes rearrangements between genomic loxPsym sites that can lead to translocations, inversions, and deletions of genes. Thus, random genetically diverse libraries are created. If a heterologous gene flanked by loxPsym sites is provided at the time of recombination, this may become inserted at the expense of non-essential genes if it provides a growth advantage [22]. The SCRaMble method has also the potential to create minimal genome sets by generating an increased number of deletions over time [23].

2.2. Meganucleases

Meganucleases (called homing endonucleases) are a large family of DNA nucleases found in a number of organisms like phages, bacteria, archaebacteria, and various eukaryotes [24]. Meganucleases were initially found to be encoded by mobile introns, and, since then, they have been repurposed for creating targeted DSBs within genomes. Their relatively small size (~165 aa) and large DNA recognition sequence (~18 bp) have made meganucleases an attractive option for genome engineering [25-26]. The LAGLIDADG family is the largest, most extensively studied, and best characterized meganuclease family in the yeast [27]. These include the mitochondrial intron-encoded I-SceI [28], the intein PI-SceI [29], and the HO endonuclease involved in mating-type switching [30] were shown to possess endonucleolytic properties that trigger homologous recombination in yeast [31]. However, the presence of a meganuclease cleavage site on the target locus is essential for meganuclease-mediated genome engineering. Therefore, if the recognition site was absent, it is required to be introduced. It thus becomes necessary to resort to the novel, artificial and programmable meganucleases, for which specificity has been tuned according to target choice, allowing high-precision genome editing) [29].

2.3. Programmable endonucleases

Programmable nucleases produce site-specific DNA double-strand breaks (DSBs), which during repair, can be used to introduce site-specific point mutations, insertions, and deletions into the genome. The genome

engineering/editing technology is evolving rapidly and includes zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas (CRISPR-associated) system as genome engineering tools. These chimeric nucleases are composed of programmable, sequence-specific DNA-binding modules linked to a nonspecific DNA cleavage domain [29-32]. Though their application for genome editing in yeast is still in its infancy, yeast is often used as a screening or testing system for enzyme efficiency [31-33].

3. Constructing biosynthetic pathways

Understanding biosynthetic pathways of high-value metabolites is crucial for the construction of yeast cell factories to produce these compounds. Several strategies have been developed for pathway identification, resulting in the elucidation of key genes of multiple high-value metabolites biosynthetic pathways [11]. A long-standing challenge in metabolic and genetic engineering is determining whether to improve the isolated host's production capacity or whether to transplant the desired genes/pathways into the host. Traditional metabolic and genetic engineering is the use of engineering principles for constructing functional, predictable, and non-native biological pathways de novo to control and improve microbial production. There are now many such examples of the successful application of synthetic approaches to biosynthetic pathway construction, for instance in the microbial production of fatty-acid-derived fuels and chemicals (e.g., fatty esters, fatty alcohols, and waxes), methyl halide-derived fuels and chemicals polyketide synthases that make cholesterol-lowering drugs and polyketides made from megaenzymes that are encoded by very large synthetic gene clusters [8]. Many secondary metabolites comprising terpenoids, alkaloids, steroids, ginsenosides, and carotenoids have been synthesized by yeast cell factories based on pathway identification and synthetic biology tools [11]. After the biosynthetic pathways are assembled, the expression levels of all the components need to be coordinated to a maximum metabolic flux and accomplish high product titers. Synthetic biology efforts to create and characterize more reusable, biological control elements based on promoters for predictably tuning expression levels. Further to this, synthetic biologists have devised a number of alternative methods for biological pathway balance, ranging from reconfiguring network connectivity to fine-tuning individual components.

4. The potential applications of synthetic biology for winemaking

The quality of excellent wines is dependent on the grape variety, climate, soil, and landscape as well as technology, innovation, and craftsmanship. Considering this, to apply the synthetic biology procedures in yeast for winemaking, one should need better knowledge about the organism and methodologies such as genetic transformation, selection markers, specific plasmid vectors, different promoters, and advanced molecular biology techniques [34]. All these tools are easily available for *S. cerevisiae* strains

which have been extensively used in food and beverage production and are generally recognized as a safe source. Concurrently, synthetic biology tools have been developed for engineering yeast as cell factories for the production of high-value secondary metabolites for medicine and industrial products [35]. The gain of knowledge from earlier work on yeast synthetic biology for drug synthesis has given the right impetus to attempt the principles of synthetic biology for superior quality wine satisfying market demands and customer endorsements.

Recently, demand for quality wine has increased, therefore to be successful in the modern marketplace, a winemaker must integrate the artistic and economic aspects of wine production, and possess a solid understanding of the intrinsic and extrinsic factors that underlie purchase motivation [36]. Wine can provide various benefits such as unique flavor [37] health, nutrition, texture, and safety (shelf life), as well as offers analgesic, disinfectant, and profound mind-altering effects [38]. Currently, active research on the role of yeast in wine fermentation has been focused on the improvement of wine sensory quality for the consumer, and obtaining unique signature flavors that help to distinguish a product from others on the market. Besides these, reducing alcohol levels, enhancing taste, high nutritional value, and incorporating novel characteristics for a better commercial value, may in fact be the key factor that defines a successful food product. The increasing preference for designer wine, beer, or cheese among foods will continue to grow among affluent consumers as they refine, improve, and discover their tasting abilities all around the world. These discerning consumers select products based on taste, preferring to pay more for a refined sensation, rather than less for quantity [39].

Against this backdrop, remarkable recent advances in biological sciences have helped and contributed immensely to understanding and deciphering the mechanism of yeast action in wine preparation. Targeted gene transfer from one organism to another related or unrelated organism, possible with recombinant DNA technology, has changed the scenario of biological research substantially. Genetic-engineered synthetic yeast is rapidly being developed which deals with enhancing wine flavor, nutritional improvements (Phenolics, vitamins), reducing toxic substances (ethyl carbamate), and improving the winemaking process by excretion of macerating and pectinolytic enzymes (Fig. 2). However, the successful commercialization of semisynthetic wine yeasts will depend on a multitude of scientific, technical, economic, marketing, safety, regulatory, legal, and ethical issues [40].

Simultaneously, considerable progress has been accomplished in yeast biotechnology which has been judiciously employed in overcoming various biological and technological problems connected with yeast fermentation.

Concurrently, the current progress in synthetic biology has been directed to design and construct novel and innovative biological modules complementary to conventional metabolic and genetic engineering efforts for building practical, expected, and non-native biological pathways to control and improve the role of living organisms in various metabolic processes. Since the yeast genome has been sequenced and in conjunction with knowledge from synthetic biology will be useful for designing rational yeast metabolic engineering, identification of target genes, and regulation of network balancing and expression levels of yeast genes. Additionally, the synthetic biology method will accelerate the assembly of various genes as well as their expressions and identify metabolite intermediates for improving commercial wine fermentation which has allowed the successful creation of an increasing number of yeast cell factories for making of high-value metabolites.

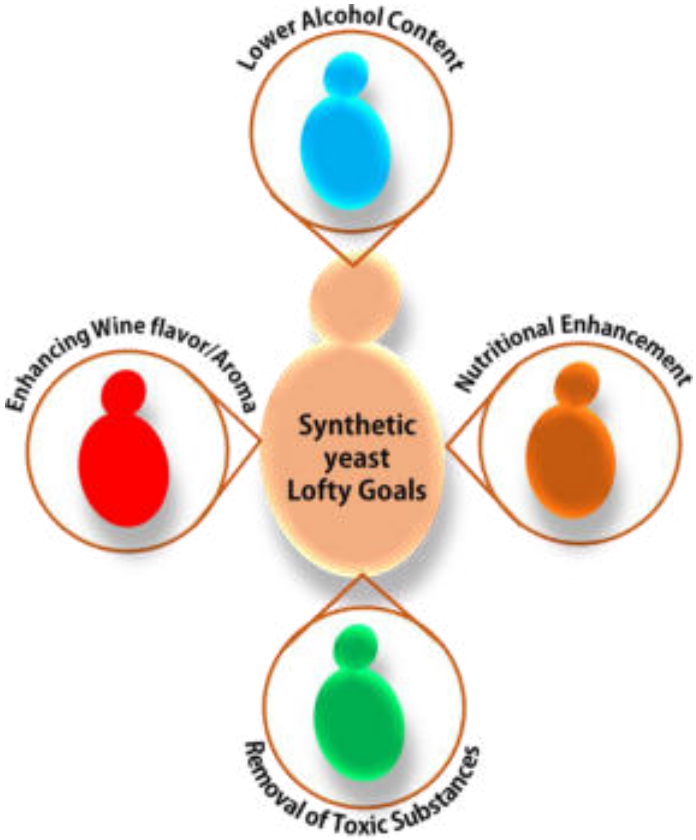

Fig. 2: Designing the yeast for ideal wine.

Furthermore, once the customized yeast is synthesized that will lead to an understanding of yeast biology, and the understanding functions of different genes, which will help to build minimal yeast genomes consisting solely of essential genes by removing unwanted genes [41]. Besides, serving as a toolbox to build minimal yeast cells for winemaking, the customized yeast with

the SCRaMbLE system will offer a greater genetic diversity for more efficient fermentation and give specific characteristics to the wine.

4. CONCLUDING REMARKS AND FUTURE OUTLOOK

Recent advances in the 'omics' technology in yeast pave the way for the understanding the yeast systems biology which provides the basis for yeast synthetic biology. Research advancements in the whole genome synthesis, by using genome reading (sequencing), writing (synthesizing), and editing (modifying) technologies along with high throughput yeast genetic engineering methods are led to the construction of synthetic yeast genome with desirable properties. The emerging technologies related to synthetic yeast biology have incredible and tremendous applications as evident from the work currently underway across the globe. As we further understand yeast systems biology, and as the availability of standardized genetic parts increases, we will better understand how to design synthetic systems in yeast with respect to winemaking. In the future, it may even be possible to use customized synthetic yeast with improved efficiency and precision in the winemaking industry by offering greater genetic diversity for the development of specific characteristic wine, otherwise difficult to obtain by conventional methods, which opens a new era of yeast synthetic biology in winemaking.

However, for any biological system, the technology used in order to take advantage of the system to its highest potential, it is crucial to know a thorough understanding of technology. A small number of biotechnology-derived technologies are approaching commercialization, but these are the minority that have met the technological challenges, cleared the regulatory hurdles, and overcome inertia in the biotechnology industry. Meanwhile, the yeast synthetic biology research raises questions about its future use in winemaking technologies and their perception by society. Yeast synthetic biology aims at overcoming the existing fundamental inabilities by developing foundational technologies to ultimately enable systematic forward engineering of biology for improved and novel applications.

It is expected that by exploiting Nature's molecular blueprint for creating genetically diverse yeast strains, synthetic biology can be used as a powerful platform for building new ultimate yeast strains.

ACKNOWLEDGEMENT

VAB thanks Indian National Science Academy for an honorary scientist fellowship.

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Therapeutic Antibodies: The Challenges and the Implications of Novel Antibody Technology

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ABSTRACT
Therapeutic antibody technology governs the biologics industry, and there is still a lot of profit to be gained by creating innovative and better antibody treatments. Monoclonal antibodies (mAbs) have risen to prominence in the area of a specific target therapy and diagnosis over the last ten years. The growing interesting monoclonal antibodies (mAbs) stems from their high binding efficiency (affinity and specificity), as well as the original molecular and structural principles that control associations with their cognate antigen. Antibody production has seen several notable developments in recent decades, but needs further improvements. This review describes the range of issues and concerns encountered in the development, manufacture, manufacture, and synthesis of therapeutic antibodies, such as safety, bioavailability, and immunological interaction, considering the growing interest in developing antibody therapies. We focus here on how technology is progressing to solve these issues, emphasizing the main antibody-engineered forms that have been modified. Besides that, the implications of novel product innovations, such as nano-carrier delivery structures to produce pulmonary delivery formulations. Ultimately, we have added recent advances in analytical methods to systematically prepare antibodies with modulated functions in depth.

KEYWARDS
Antibody engineering, Monoclonal antibody formulation, Therapeutic antibodies.

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1. INTRODUCTION

Antibody therapeutics is the speediest drug group on the market, attributable to numerous biologically advantageous properties like specificity, efficacy, and metabolic stability. The production of these essential drugs has also benefited from developments in humoral immunology and protein engineering. Actively, 76 antibody-based therapeutics are currently employed in the clinical trials, and many more in the final stages. Antibodies are most valuable in the areas of oncology and immunology to solve the dynamic pathobiology of disease and develop current treatments, managements and progressively novel antibody variants have replaced the traditional monoclonal antibody over time [1].

Antibody therapeutics have been and continue to be the most potent and vital biological therapeutic system in the pharmaceutical industry since the 1st therapeutic monoclonal antibody (mAb) Orthoclone OKT3® (Janssen Biotech, USA) was authorized by the US-FDA (Food and Drug Administration) in 1986 [1, 2]. Therapeutic antibodies deal with a variety of conditions involving tumors, pathogens, and cardiovascular and neurological disorders [3]. The antibody therapeutics system as a whole has been recognized as the most exciting class of pharmaceutical technology to date; it is constantly being focused on the new biological targets and introduced in a variety of settings to generate precisely engineered next-generation antibody therapeutics, also known as "biobetters" [4].

Antibody engineering has progressed considerably resulting in the highly developed, tactically formulated biobetter therapies, as well as biosimilarm Absoriginator. Antibody-drug conjugates (ADC), bispecific, isotype-switched, and glycoengineered whole mAb formats have been formatted. These were chosen for their excellent efficacy, capacity to interact with dual biological targets and modulation of the Fc effector functions. The crystallizable fragment (Fc), antigen-binding fragment (Fab), and single-chain variable fragment (scFv) are mAb fragments with important features such as high biological selectivity and immunological activation. The key part of innovation in creating the next wave of biobetter therapies has been the extraction of these fragments for integration with the other mAb segments, biologically active proteins, cytotoxic drugs, or drug carriers (Figure 1) [4].

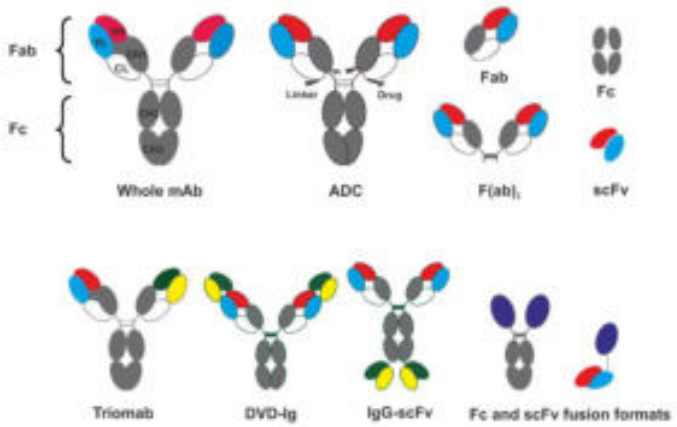

Figure 1: Schematic diagram of the whole monoclonal antibody (mAb), the fragment fragmentation of the mAb, and the notable mAb formats for tactical therapeutic applications. Fragment formats comprise crystallisable fragments, (Fc), antigen-binding fragments and single-chain fragments(scFv) (Fab and F(ab)$_2$). Additional whole mAb formats included ntibody-drug conjugate (ADC), triomab, DVD-Ig, and immunoglobulin–scFv fusion (IgG-scFv).

2. EVALUATION OF THE THREATS AND CONCERNS IN PRODUCTION

Therapeutic whole mAbs need a mammalian expression system to generate the biologically functional molecule; nevertheless, a portion of mAb and recombined fusion mAb are appropriate for lower organism expression systems, with enhanced (or deficient) glycosylation [5]. In contrast to the chemically synthesizable oligopeptides, wholetherapeuticallymAbs are significantly larger (with monomers between 140 and 160 kDa), consisting of four peptide chains (two heavy and two light chains) linked by disulfide bonds and affiliated non-covalent associations. Furthermore, antibodies comprise Fc (N297) glycosylation in a preserved Fc region, contributing to its stability and the activities of the immune effector.In addition to the processing of peptides, glycosylated, folded, oriented, and covalent binding of the peptide chains of antibodies are needed to generate the entire biologically functioning antibody material. It is commercially unrealistic to generate a biologicallyfunctional whole mAb product by synthesis through chemicals and in organism expression platforms, such as bacteria, yeast, insect, and plant cells, and the machinery to generate equivalent tertiary structures and glycosylation profiles may be inadequate. Numerous industrially important bacteria such as *E. coli* have flawed machinery for adding post-translation glucose; yeasts that cause immunogenic hypermannosylatedglycans; and insect cells insufficient in sialylation machinery and develop immunogenic glycan complexes [5]. In *E. coli*, a mAb product is not optimum for the processing due to the poor efficiency and difficult culture conditions that facilitate product degradation. The protein production is thus intracellular as the integration and extraction of mAb's include more processing steps such as cell lysis, recovery of the body inclusion, solubilization of protein and renaturation before further downstream processing stages [6]. Notwithstanding these drawbacks, the production of lower organism expression systems is generating a lot of attention because of the simpler culture conditions, lower media requirements, faster organism growth, and higher product output, especially in comparison to other expression systems [5].

Considering the specifications in the system of mAb discovery, production, formulations, and therapy, many biggest hurdles that emerge have created immense bottlenecks. Like other bio-therapeutics, the manufacture of mAbs and mAb-based therapeutics is confined to cell-based expression systems, which are comparatively costly and uncomplicated, can generate different results depending on the product and expression system, and needs further processing to extract biologicalcontaminant from the expression system. While affinity chromatography is a powerful technology for the initial harvest ofmAb for processing, collection and other downstream methods, such as viral inactivation, apply mAb to harsh pH and salt conditions, which can chemically degrademAb, resulting in the product instability and loss [7]. The glycosylation and heterogeneity of mAbs are influenced by several factors during the production process, which impacts their biophysical and pharmaceutical

properties. This does not directly tackle these deviations; mAb processing technologies are improved and regulated to minimize variability and off-targetedcytotoxicities.

To ensure a desirable bioavailability, mAbs and mAb-based therapeutics are presently limited to lyophilized and liquid-based formulations for intravenous (IV) or subcutaneous (SC) delivery, as is the case for all other bio-therapeutics. The restrictionsare triggered by protein self-association and intrinsic stability, as viscosity and the propensity to accumulate which are dependent on mAb concentrations. Formulations are designed to obtain the maximum dose concentration at the smallest possible injection volume while maintaining the efficacy of the mAb [8]. Because of their solubility, self-association, and aggregation characteristics, viscosity mAbcontinues to be a major constraint for preparing as an SC administration; some mAb therapies are appropriate and others are not. Alternative non-administration techniques, such as pulmonary delivery, apply compressive damage to the mAb, making it more unstable and prone to the degradation. Furthermore, due to the chemical and enzymatic breakdown, and poor absorption in the gastric and intestinal regions, oral delivery administration is ineffective [7, 9]. Figure 2 shows a brief summary of therapeutic mAb production progresses through the various phases of the concept.

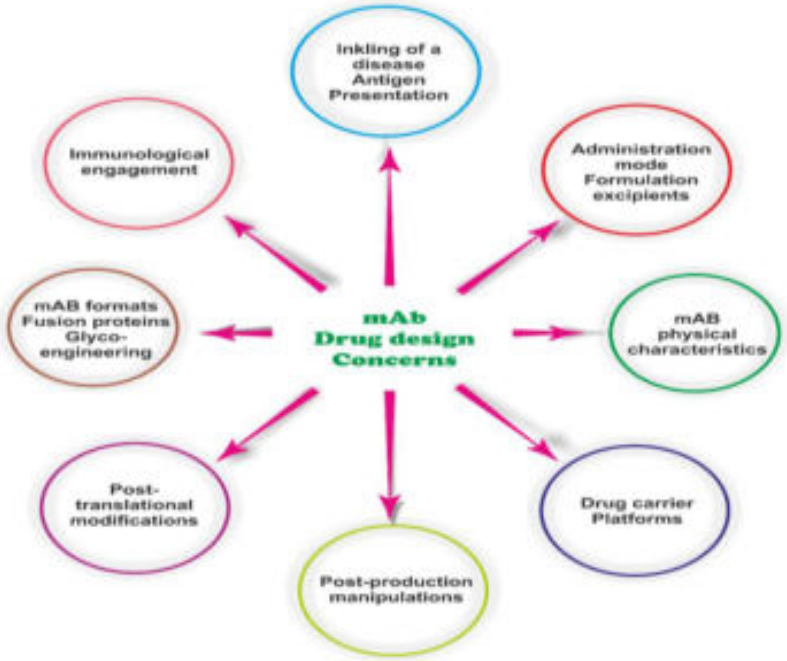

Figure 2: In designing and manufacturing mAb-based therapeutics, flowconcern flows from one process to the next in a conceptual representation of the model stages of mAb drug production.

3. TECHNOLOGIES FOR mAb REVELATION AND PRODUCTION

Conventional therapeutic antibody development technology involved immunizing animals, specifically mice, with the desired antigen to generate a mixed population of B lymphocytes that can generate antibodies against the antigen. B lymphocytesB lymphocytes would be extracted for immortalization, resulting in monoclonal hybridoma cell lines that naturally begin producing antibody targets of interest, which would then be screened using display technologies to extract the possible targets [7]. In contrast to whole human mAbs, this approach resulted in the production of extremely precise nonhumanmAb repositories that were possibly immunogenic and less effective at evoking an immune response. Technologies emerged to resolve immunogenicity by grafting variable domains (chimeric) or complementarity-determining region (CDR) fragments (humanized) extracted from lead nonhuman antibodies to a human antibody system, resulting in the chimeric and humanized antibodies. To boost the humanization technique, transgenic mice were produced with identical human genes replacing the murine antibody of heavy and light chain genes, resulting in entirely human antibodies for further developments [4, 7]. It efficiently couples the expression of mAb proteins to the genes that encode them for the panning of high affinity leads, and phage display technology remains the most popular screening technology for antibody gene pools for sensitivity to a targeted antigen. Antibody variable genes are extracted from B-lymphocyte pools using polymerase chain reaction (PCR) and cloned into a phage expression system that displays the expressed mAb on the phage's exterior. The database of vectors is then retrieved in phages and tested against a particular target antigen. The variable genes of the target mAb candidates can then be extracted and sequenced for the creation of anmAb drug design and the creation of an appropriate expression platforms. Phage pools go through multiple cycles of sorting to extract high-affinity candidates, and the variable genes of the target mAb candidates can then be extracted and sequenced for the creation of a mAb drug design and the production of a reasonable expression system. Display technology has driven the development of tremendous repositories for the target affinity screening, owing to error-prone PCR-based mutagenesis. Since glycosylation places incorporated in CDRs are expressed via this system, yeast interface display enhances these screening technologies [7].

In hybridoma cell lines, mAb function, output, and effectiveness are different. For the market needs high effectiveness, flexibility, consistency, and replicability of expression and high-distribution mammalian expression methodologies have been introduced. Antibody genes of concern are inserted into extremely effective mammalian cell lines for antibody production and efflux; a mAb can then be explicitly harvested from the culture supernatant employing affinity chromatography. Presently, for mammalian expression, Chinese hamster ovary (CHO), mouse

myeloma (NS0), and mouse hybridoma (Sp2/0) cell lines are used in industrial whole therapeutic antibody expression systems. However, for initial research, transient expression in human cell lines such as embryonic kidney (HEK 293), amniotic (CAP), a combination of HEK 293 and lymphoma (HKB-11), and embryonic retina (PER.C6) is preferred to steady CHO expression [5, 10]. To eliminate possibilities with low manufacturability qualities, the developed mAb library is subjected to specific in vitro testing as well as composition stabilization screening [11]. Leading mAb contenders that show promise for further research will be designed for high yielding, steady expression, and more thorough analysis in the run-up to therapeutic production. The disparity in glycosylation profiles between human and hamster expression systems is, nevertheless, a disadvantage. CHO-produced mAbs contain the immunogenic non-human glycoform Neu5Gc and have a higher sialylation ratio, which may contribute towards less antibody-dependent cellular cytotoxicity (ADCC). Until submitting to industry scale-up, leads must be detected and thoroughly defined in the expression systems to be built for commercial productions in the initial phases of the process [5, 12]. Authorized Fc fusion therapeutics dulglutide (Trulicity®, Eli Lilly, Indianapolis, IN USA), efmoroctocogalpha and eftrenonacog alpha (Eloctate® and Alprolix®, Biogen, Cambridge, USA), among others, are produced in a HEK 293 expression system that leads to the recognition of human-based expression systems for the development of mAb- HEK 293 expression systems, on the other hand, are vulnerable tomAb aggregation in cultures, which is harmful to the cell viability and results in yield reductions during manufacturing. As a result, HKB-11 and PER.C6 are the commercial human cell lines for the expression of mAb expression [5, 13].

The advancing in-vitro cell-free synthesis technology with the bacterial and CHO cell lysates are being established, which can alleviate the types of unwanted biologic by-products since the cell lysates machinery expresses purely the protein generated by the mAb genes in the method. This latest technology does not apply to manufacturing at the industrial levels, it is very promising as an alternative to the live cultures. For example, preservation of culture and highly defined media areno longer required, reactions may persist, lysates can be reused with high reproducibility, unmodified linear DNA (reducing the need for multiple cloning steps), and external enzymes can be incorporated into the system to design certain post-translation changes [14, 15]. Notwithstanding the benefits for mAb production from this technology, cell lysates face the same issues as their host cell expression system. That is, the endogenous system of that host organism limits post-translational alterations to the mAb products from lysates from the lower species. Furthermore, mammalian cell lysates are difficult to prepare and their yields are comparatively poor [16, 17].

To increase the manufacturability of mAbs, modern bioprocess engineering and cell line growth techniques have been critical. Many existing mAb expression platforms use commercially formed CHO cell lines for the increased stability preference by tolerance to methotrexate and methionine-sulfoximine suppression, with dihydrofolate reductase or glutamine synthetase deficiency [18]. In the absence of serum from growth medium for large-scale fed baths, perfusion systems or continuous culture systems, mammalian cell lines have been optimized for the suspension growth as they embrace higher cell densities and mAb-titres. Furthermore, cell lines for strengthened metabolic processes are being developed continuously, adding glycosylation mechanisms, higher secretion,and tolerance toapoptosis for longer persistence in instances to generate super-producing cell lines that can maintain a continuous culture [19].

The media is being continuously fed into the culture system during the manufacturing phase of feed-batch, infusion and continual growing, while parameters like cell viability, cell density and metabolite level are being regulated in real-time until the variables showed that it is the best ready to collect the released mAb from the supernatant culture. The distinction in the perfusion and continuous cultivation processes in the cell dilution rate in a continuous culture is designed to continue at the rate of cell growth that enables the continuance of mAb expression and needs constant harvesting of the supernatant [20], which is the same as or higher than a cell growth rate. The extraction of supernatant involves technology for confirmation, such as precipitants/flocculants application (for example, polyethylene glycol, diethylaminoethyl dextran, caprylic acid and polyethylene), the utilization of high throughput centrifuges and the removal of cells and recyclable filtering methods to reduce the workload for a mAb to be collected by affinity chromatography. The mechanical strain is inevitable, although they can result in mild mAb by fragmentation and aggregation.

4. ISSUES TO CONSIDER AND TECHNOLOGIES FOR FORMULATION

As with other biotherapies, finding new ways to formulate mAbs and mAb-based methods are difficult challenges. First, whole therapeutic mAbs are in appropriate, as they are vulnerable to chemical and enzymatic breakdown in the gastrointestinal tract for non-invasive oral, nasal, or pulmonary routes of administrations. Due to the mAbs' polar exterior charge and a significantly larger MW (Molecular weight), mAb's bioavailability across these route options is low, restricting transportation across the mucosal membranes. Fragment mAb operating systems have been established in conjunction with the excipient and PEGylation technologies to explicitly avoid pulmonary transport restrictions. However, the physical stresses introduced to mAb pose more problems of instability of mAb, leading to the decay

and reduction of efficiency. [21, 22]. Few pulmonate-based biologics have beeneffectively authorized, noticeably Afrezza® (Mann Kind, Westlake Village, CA, United States) insulin formulation and, presently, erythropoietin-Fc fusion and a nanobody addressing respiratory syncytial virus are in the medical tests, which have promised the further creation of both systemic and localized mAb management strategies. Various drug carrier innovations such as microencapsulation, liposome and nanoparticles potentially improved stability and monitor mAb releases that can extend further their half-life. These nanocarrier formulation strategies are of great importance, as they continue to promise to produce less destructive inhaled mAb-based formulations with characteristics superior to existing formulations [23].

Through the utilization of excipients to accelerate solubility, minimize viscosity, and boost the stability of mAbs, injectable mAb formulations can be further advanced. For formulations based on their physical characteristics, pharmacokinetics and reliability, excipients are perceived. For example, polysorbates are usually used as a stabilizing agent in the biological products; however, they can add denatured proteins at large concentrations and trigger negative consequences such as injection site reactions [24]. Injection-based mAb formulations are co-formulated with recombinant human hyaluronidase, especially as a booster of permeation for more effective tissue absorption, but the introduction of this external biologic adds subsidiary stresses to the formulation's viscosity and aggregation tendency [6, 22, 23].

Antibody therapies for IV (intravenous) administration are formulated as lyophilized powders that must be reconstituted and further diluted, while injectable administrations are formulated as liquid-based solutions in pre-filled syringes. When contrast to the lyophilized formulations, liquid formulations of mAbs are more vulnerable to physiochemical degradation, are less stable, and have a shorter shelf life. The drying techniques used to make lyophilized preparations, on the other hand, subject the mAb to physical stresses that cause instability and degradation, resulting in the decreased effectiveness [24].

5. TISSUE PENETRATION OF MONOCLONAL ANTIBODIES FOR CANCER TREATMENT

For optimum bioavailability and systemic dissemination, antibody therapies are presently confined to invasive parenteral routes of administration; nevertheless, transmission to target tissues, such as tumors for cancer treatments, remains a concern because of the polar surface charge and significantly larger MW of mAbs, accessibility to tissue from blood vessels is low, restricting transportation through physiological barriers. Tissue absorption efficiency is also affected by systemic and local mAb clearance rates. The ability of mAbs to bind to their biological targets has

been improved through maturation and strategic mutation technologies, resulting in faster mAb diffusion rates and improved efficiency. However, the potential binding barrier, in which the antigens expressed on the tumor's periphery of the tumor absorb the significant proportion of the mAb delivered to surrounding tissue, limits the accessibility totumors; this effect is aggravated by over expressed antigen. There is little or no change in tumor diffusion concentrations, tissue penetration, or aggregation when the affinity of the whole mAbs is increased beyond 1nM [25]. The enhanced affinity of mAbs for cellular targets contributes to elevated mAb absorption, internalization, and catabolism, lowering ADCC and increasing mAb clearance rate. This mechanism is used to target the delivery and liberation of potent drugs, resulting in programmed cell death [26].

Fragment mAb mechanisms have demonstrated better tissue absorption and biodistribution than whole mAb therapeutics; furthermore, the in vivo half-life and retention periods of small fragments lacking an Fc region are significantly decreased. PEGylation, as well as strategic Fc mutation and glycosylation engineering, have been used to increase neonatal recovery from the Fc receptor (FcRn) and boost the half-life of mAb fragments. Hyper-glycosylation technology has also been used effectively in other biotherapeutics, such as Aranesp®, glycosylated erythropoietin (Amgen, Thousand Oaks, USA). Nanocarrier systems that are larger than the entire mAb, on the other hand, have shown excellent pharmacokinetics and tumor retention, as well as the previously identified strengthened stability and persistent, regulated discharge of mAbs [23]. These improved characteristics have sparked concern in establishing mAb-nanoparticle platforms for controlled administration for better tumor penetration and efficient and stable therapeutic drug delivery. Supplemental formulation strategies designed for sustained delivery of mAbs, in contrast to nanocarriers, comprise hydrogels and crystalline antibodies, which have demonstrated success as stable injectable formulations for production [6].

6. EFFICIENT MODULATION OF mAb IMMUNE EFFECTOR FUNCTIONS

Alterations of mAb effector functions through iso type switching, glycoengineering, and strategic mutations have proven beneficial in the advancement of more efficacious mAb treatment methodologies. Antibody binding to Cq1 facilitates the complement cascade, while antibody binding to $Fc_{\gamma}R1A/B$, $Fc_{\gamma}R2A$, and $Fc_{\gamma}R3A/B$ receptors stimulate immune effector activities; $Fc_{\gamma}R2B$ neutralize the effector response, and FcRn increases the half-life of mAb.The linkage to these receptors is mainly performed through sites in the Hinge, CH2 and the Fc preserved glycosylation region (N297). IgG3 does not attach to FcRn effectively, which decreases its half-life to about seven days, compared to a 21-day half-life for the

other IgG isotypes. In most cases, a prolonged half-life is better favoured in order to sustain the effective dose of a mAb in serum [27]. IgG2 and IgG4 mAbs have a lower effector activity than IgG1 mAbs and are therefore used in circumstances where minimal immune system involvement is substantiated to boost the safe operation ofmAb therapy—for example, with ADCs lowering off-target cytotoxicity. Eculizumab (Soliris®, Alexion Pharmaceuticals, New Haven, CT, USA) is the first (and currently the only) accepted hybrid whole mAb of recombinant IgGκ2(C_H1/Hinge)–4(C_H2/C_H3) hybrid whole mAb. More cross-subclass variant mAb treatments are being developed, with key amino acid substitutions from the IgG2 and IgG4 subclasses being incorporated for purposefully suppressed effector functions (Wang et al., 2018 [29]). The elimination of the conserved glycosylation site via amino acid substitution of N297 or T299 (aglycosylation) has also shown reduced effector function; even so, this occurs at the cost of mAb consistency and is thus not a viable technique for whole mAb therapies [27,28].

Boosting ADCC and CDC, on the other hand, is effective for attempting to engage the immune system in tumortissues in the absence of a conjugated cytotoxic drug. Certain glycoengineered alterations to the conserved glycosylation area in the Fc, such as core fucose deficiency (afucosylation) and hyper-galactosylation, have shown improved mAb binding to Fc R3A and Cq1, especially for a boosted ADCC and CDC effect [29]. Benralizumab (Fasenra™, AstraZeneca, London, UK) and mogamulizumab (Poteligeo®, Kyowa Hakko Kirin, Tokyo, Japan), as the well as low fucose-content obinutuzumabmAb (Gazyva®, Genentech, San Francisco, CA, USA), with many more in the production demonstrating the financial stake. Moreover, the importance of manipulating sialylation in mAb therapeutics is debatable, with some studies showing a significant decrease in ADCC and CDC and others showing no substantial differences. This demonstrates the critical need to characterize and interpret the in vivo effectiveness of such manipulation [29,26]. There are presently no authorized mAb therapies that include amino acid substitutions for strengthened half-life via amplified binding characteristics to FcRn, strengthened CDC via binding to complement factor Cq1, or boosted ADCC via increased binding affinity to Fc_γR1A and 3A. However, some mutations have been identified, patented, and are in the clinical trials, indicating commercial attention in this technology to obtain better mAb therapeutics [29].

7. COMPUTATIONAL METHODS FOR PREDICTION AGGREGATION AND CONCETUAL DESIGN OF mAbs

The progression of in silico analysis of mAb peptide sequences, structures, conformation, and their associated biological engagement has been crucial for the development of numerous computational tools for characterizing, designing, and

optimizing mAb-based therapeutics. The production and the sustained quest of mAb structural data for in silico analysis have resulted in the advancement of several integral databases, most noticeably IMGT®, which serves as a vital resource for data mining [30]. Molecular dynamic (MD) simulation analysis has powered the computational analysis of pertinent molecular interactions within the mAb molecule, mAb binding to biological targets, and mAb surface connection with the external environment. These interactions can be used to deduce the mAb's intrinsic stability, target binding associations, solubility, and aggregation proclivity. MD simulations and free energy calculations from crystal structures are critical for the extremely precise elaboration of mAb-target intermolecular interactions that correspond to binding affinity, as considerable interactions such as hydrogen bond establishment can be forecasted and the potency of the molecular associations can be determined [31].

Moreover, the development of computational modelling and simulation tools that concurrently evaluate the topography and surface polarity has fuelled the elaboration of mAb self-association, solubility, and aggregation propensity. Noticeably, AGGRESCAN3D, TANGO, and PASTA are the most prolific tools to predict the site-specific aggregation propensity of mAbs [32]. The preliminary explication of mAb structural data amino acid sequences and higher-order structure (HOS) is experimentally inferred to develop crystal structures that model thesolid-state 3D structure. Mass spectrometry technologies have grown to generate high-throughput and orthogonal analysis to explicate mAb peptide sequences, oxidation, deamidation, and glycosylation heterogeneity, and more recent times, to explicate native, destabilized, and aggregated HOS [33]. X-ray crystallography technologies have been the mainstay for explaining the crystal structure of mAbs. Due to the intricacy of mAb HOS and the degree of mAb conformational heterogeneity, generating crystalline mAbs is difficult. Interestingly, several complementing technologies for determining structure and interactions have emerged in the last few years, including circular dichroism (CD), infrared (IR) and Raman spectroscopy, cryogenic-electron microscopy, and nuclear magnetic resonance (NMR) spectroscopy [29]. The highest sensitivity and local specificity are provided by 2D-NMR and X-Ray crystallography (followed by MS) among the technologies accessible for HOS explanation. On the contrary, CD, IR, and Raman are high-throughput methods with much lower sensitivity. Only four whole IgG antibodies have had their crystal structures dictated (PDB IDs: 1HZH, 1IGT, 1IGY, and 5DK3), with 1HZH as a wholly human IgG1 and 5DK3 as a humanized IgG4, both of which have been utilized as model structures for MD simulations (Kumar et al., 2018). Furthermore, fragment mAbs have a much higher success percentage in developing crystal structures, which has spurred a lot of interest and participation in producing

mAb fragments and targeting complexing crystal structure libraries for data mining and in silico analysis [34].

Computational modeling tools that particularly characterize the topography and surface polarity of mAbs, as well as the local spatial configuration of amino acids in the structure of mAbs, solvent accessibility, and local surface charge, are used to assess further self-association, dissolution rate, and aggregation tendency [6]. Surface-exposed hydrophobicity is sought as the leading mechanism for protein self-association driving aggregation tendency, with aggregation-prone regions (APRs) discovered in the mAb structure. Demonstration of resistance to aggregation and improved solubility of discovered APRs in gatekeeper (i.e., polar) amino acids (i.e. polar) confirms this for increasing the stability (van der Kant et al., 2019 [35]). Most APRs found are in physiologically active mAb regions, specifically CDR and Fc. Addressing these APRs mainly through mutation may affect essential biological activities and mAb structure. Analyses of mAb intermolecular contacts are required to validate the intrinsic stability of substituted mAb variants; if conformational fluctuations are discovered, the physiologically active interfacesare likely to be disrupted. Interestingly, changes aimed at improving biological functions have been shown to have a negative influence on mAb absorption and stability, implying that the self-association nature of mAbs is connected to its biological functionality. In addition todirect substitution in mAb structures, isotype switching and targeted insertion of N-linked glycans have been documented to significantly interfere with the effects of APRs [36].

Additionally, although N-linked glycosylation in the CDR sections of mAbs is unusual, it has been shown to increase mAb solubility and stability characteristics without affecting target affinity when contrasted to the same mAbs with eliminated CDR glycans [37-38]. The rationale for selective glycan insertion is predicated on the selection of N-linked glycosylation sites that are spatially close to APRs, with the added glycan sterically limiting the APR from self-association interaction. The advantages of prolonging mAb half-life with strategic glycan additament, as seen with hyper-glycosylated biotherapeutics (as well as boosting mAb solubility and stability for better synthesis techniques), have yet to be realized, implying a very interesting and highly pertinent technology for further investigation [41].

8. CONCLUSION AND FUTURE PROSPECTIVE

Therapeutic antibodies have developed as a significant, prominent technology in the biopharmaceutical sector. Antibodies have been repurposed in a variety of formats, allowing them to be used to design and customize highly specialized treatments such as ADCs as a specific target drug distribution system, bispecific and fragment mAb platforms for geared participation and elevated bioavailability, and recombinant Fc

fusion proteins for elevated half-life and immunological interaction. Further developments encompass modification of Fc effector functions via Fc manipulations, such as isotype switching, glycoengineering, or selective mutations in the Fc region, as well as PEGylation of fragment mAbs for increased half-life. Advances in research, manufacturing, and formulation technologies have contributed to the effectiveness of therapeutic antibodies, most significantly through the establishment of expression systems and the switch from IV to SC formulations. Human-based expression systems have been widely exploited in mAb research and are rapidly becoming a standard production platform for mAb therapies. Moreover, cell-free synthesis technology opens the possibility of greater efficiency in the manufacturing process.

Upcoming generation antibody treatments have resulted in significant improvements in therapeutic outcomes, several aspects of the manufacturing process and formulation development strategy must be explored. Because antibody-based therapies are sensitive to chemical and enzymatic destruction via oral, nasal, or pulmonary modes of administration, they are now limited to IV or SC administration. Although optimal bioavailability via IV/SC administration, mAb-based treatments have low tissue absorption, limiting their local bioavailability and necessitating high concentrations to provide an effective dose.Stability of mAb-based therapies is a significant and recurring issue that must be addressed since it affects manufacturing yield and formulation considerations. Several techniques are being developed to increase the stability of mAbs so that formulations for pulmonary or oral delivery can be produced.

Interestingly, computational technologies have advanced, complementing experimental methodologies for predicting antibody structure and aggregation. The stability and aggregation tendency of mAb-based therapeutics have improved through rational mutation and glycosylation within the framework area, which can be translatable to all mAbs within the same isotype. Additionally, nanocarrier technologies have been found to improve the stability of mAbs and possibly control their discharge. The development of rational mAb design in conjunction with nanocarrier technology can overcome these problems, produce superior therapeutic techniques, and eventually formulate for non-invasive administration ways like as pulmonary delivery.

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Anticancer alkaloid camptothecin synthesized by cell cultures of *Nothapodytesnimmoniana* derived from endosperm explant

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ABSTRACT

Camptothecin (CPT), one of the most potent anti-cancer alkaloids, is largely obtained from the endangered plant NothapodytesnimmonianaMabb. Graham., endemic to the Western Ghats of India. Efforts were made to establish high CPT yielding cell cultures from the explant endosperm of N. nimmoniana seeds. Callus and suspension cultures were initiated from endosperm tissue of mature seeds devoid of embryos and cotyledons. Cultures were developed on Murashige and Skoog's medium supplemented with 2-4-dichlorophenoxyacetic acid (2 mg/L), 6-benzylaminopurine (0.5 mg/L) and sucrose (20 g/L) under standard culture conditions. Suspension cultures were established from endosperm derived calli in MS Basal Medium containing sucrose 20 g/L. CPT content was detected, confirmed and estimated using TLC, HPTLC, HPLC and GC-HRMS techniques. A significantly high CPT content was detected in endosperm-induced calli (0.981 mg/g dry wt.) followed by that detected from stem- induced calli (0.42 mg/g dry wt.) and leaf-induced calli (0.33 mg/g dry wt.). A noticeable quantity of CPT (0.079 mg/g dry wt.) was detected in suspension cultures obtained from endospermic calli. Endosperm-derived cultures of N. nimmoniana can be selectively used, over other explants, for large-scale production of CPT.

KEYWORDS

Callus, Camptothecin, Endangered, Endosperm, Nothapodytes nimmoniana, Suspension cultures.

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ABBREVIATIONS

BA- N^6-Benzyl adenine purine, CPT- Camptothecin, 2,4-D-2-4-Dichlorophenoxyacetic acid, GA3- Gibberellic acid, MS- Murashige and Skoog's Medium, NAA-Naphthalene acetic acid, TDZ-Thidiazuron.

1. INTRODUCTION

Nothapodytes nimmoniana Mabb. Grahm. belonging to the family Icacinaceae is an important medicinal plant endemic to the Western Ghats in India. It has been declared as an endangered plant owing to its over exploitation for drawing out the important anti-cancer compound camptothecin (CPT). CPT has been proved to be a potential anti-cancer [1], anti-HIV [2]and anti-malarial [3], [4] compound. Camptothecin, a monoterpene indole alkaloid, is a highly demanding drug used in treatment of several cancers. The huge requirement of the potent drug is currently met mostly by extraction from the wood-bark tissues of *N. nimmoniana.* Extensive harvesting of the wood bark has caused this plant to become endangered and has posed a high threat to the existing population of the medicinally important species in the hot spot areas of Western Ghats in India [5]. CPT inhibits the action of DNA topoisomerase I which is an important unwinding enzyme in eukaryotic systems and hence arrests cells at S-phase of cell cycle [6]. CPT forms cleavage complexes with the enzyme DNA topoisomerase I which leads to blockage of cellular mechanisms like transcription and DNA replication. Since tumor cells undergo continuous cell divisions, they are affected more by CPT than the normal cells of the body. CPT is therefore currently efficiently used for treating various solid tumors [7]. High demand and lack of chemical synthesis has led to a drastic reduction in the population of *N. nimmoniana* [8]. Plant tissue culture techniques have been successfully used to develop alternative methods for production of the highly expensive drug CPT from *in vitro* and *in vivo* grown plantlets [9]. CPT production has been reported from callus and suspension cultures initiated from embryo [10]. Reports have shown lesser amounts of CPT from callus cultures generated from entire seeds of *N. nimmoniana* collected from Mahabaleshwar area of India [11].

Endosperm tissue is formed by the process of double fertilization in angiospermic plants, where out of the two male gametes; one fertilizes the egg to form zygote and the other fuses with secondary nuclei to form triploid endosperm. Triploid plants are usually seed sterile but often produce varieties with high vigour and superior vegetative properties [12]. Hence, callus obtained from triploid endosperm can be useful in developing triploid plantlet varieties with certain enhanced inherent properties such as higher timber value, higher CPT content. Such endangered and high demand plants can be propagated vegetatively and multiplied easily mainly through micropropagation [13]. In the present study, callus and suspension cultures were generated from endosperm tissues of *N. nimmoniana* and a higher production of CPT from them was reported.

2. MATERIALS AND METHODS

2.1. Chemicals

All the chemicals used for this study were highly pure and of analytical grade. Methanol (HPLC grade) and pure camptothecin powder was purchased from Sigma Chemicals (St. Louis, USA). Chloroform, ethyl acetate, di-methyl sulfoxide and ethanol were purchased from S D Fine-Chem Limited. MS basal media, sucrose, clarigel, all plant growth hormones and glasswares including phytajars were purchased from Hi-Media (Mumbai, India).

2.2. Plant material

Seeds of *N. nimmoniana*were collected in the month of February-March from areas around the forests of Dajipur, Kolhapur, Maharashtra, India. Seeds were air dried and stored under 4ºC for further use. Authentification of plant materials was done by experts belonging to the Department of Botany, Shivaji University, Kolhapur. Surface sterilization of decoated seeds was carried out using 70% ethanol for 2 min followed by treatment with 0.1% $HgCl_2$ for 1 min. Every step was followed by three washes with sterile distilled water in a sterile phytajar. Surface sterilised seeds were soaked overnight in sterile distilled water containing GA_3 (1 mg/L). Surface sterilised seeds were inoculated on MS basal medium for germination under dark conditions. Some of these seeds were dissected carefully to excise the embryo along with the two cotyledonary leaves and the remaining endospermic tissues were inoculated on MS basal medium supplemented with various combinations of auxins and cytokinins (**Table 1**).

Table-1: Effect of PGRs on callus formation from endospermic tissue of *N. nimmoniana* seeds and CPT production from suspension cultures developed from them.

Auxin [mg/L]		**Cytokinins [mg/L]**		**Callus formation response (%)**	**CPT [mg/g dry wt.]***	
2,4-D	**NAA**	**BA**	**Kinetin**		**Callus**	**Susension culture**
1	-	0.5	-	27.86	T	T
1	-	1.0	-	12.30	T	T
1	-	2.0	-	11.96	T	T
1	-	-	0.5	13.67	T	T
1	-	-	1.0	24.73	T	T
1	-	-	2.0	33.47	T	T
2	-	0.5	-	67.33	0.312 ± 0.24	0.023 ± 0.17
2	-	1.0	-	**89.45**	**0.981 ± 0.02**	**0.079** ± 0.02
2	-	2.0	-	12.76	T	T

2	-	-	0.5	34.66	T	T
2	-	-	1.0	14.50	T	T
2	-	-	2.0	09.34	T	T
5	-	0.5	-	63.33	T	T
5	-	1.0	-	12.87	T	T
5	-	2.0	-	16.33	T	T
5	-	-	0.5	11.24	T	T
5	-	-	1.0	30.86	T	T
5	-	-	2.0	19.54	T	T
-	1	0.5	-	12.23	T	T
-	1	1.0	-	32.66	T	T
-	1	2.0	-	09.87	T	T
-	2	0.5	-	77.25	0.181±0.02	0.012 ± 0.03
-	2	1.0	-	42.33	0.045±0.11	0.007 ± 0.02
-	2	2.0	-	50.66	0.023±0.07	0.001 ± 0.08
-	5	0.5	-	43.89	T	T
-	5	1.0	-	66.78	T	T
-	5	2.0	-	12.56	T	T

*Intracellular CPT detected from biomass at the end of 4 weeks of incubation; Values are mean of three replicates ± S.D.; T-Trace amount of CPT detected.

2.3. Cell culture

Endosperm tissues of the surface sterilized seeds as well as stem, leaf and root explants of the *in-vitro* germinated seedling were inoculated aseptically onto MS basal medium (25 mL) supplemented with combination of various plant growth regulators (PGRs), sucrose 20 g/L and clarigel 3 g/L. PGRs such as 2,4-D, BA, NAA and kinetin were used alone and in combination in concentrations ranging from 0.5, 1, 2, and 5 mg/L. Ten explants were placed onto a petri-plate containing MS medium with respective combination of PGR and all experiments were carried out in triplicates. The cultures were incubated at 25 ± 2 ºC and 16 h light and 8 h dark photoperiod for 4 weeks. The calli formed were further subcultured for a period of 4 weeks on the same culture medium and incubated under the same culture conditions. Suspension cultures were established using soft friable calli derived from endosperm tissues. 1 g of chopped friable callus was inoculated in 100 ml liquid MS basal medium in 250-ml Erlenmeyer flasks which were supplemented with the same growth hormone combination that was used to induce the respective callus cultures. Liquid cell cultures were maintained under the same culture conditions but on orbital shaker (LabTop Instruments Pvt. Ltd., Thane, India) at 100 rpm for 4 weeks. Culture sampling was done at the end of every week for determination of biomass (growth) and CPT production.

2.4. Extraction of CPT

Cell suspension cultures were filtered to obtain the cell biomass which were further dried in the oven at 55 ºC for 12 h. The dried biomass was homogenized in methanol (2 ml/g of biomass). Methanol based extraction of dried biomass and the filtrate was repeated thrice and all the respective fractions were pooled together in 15 ml centrifuge tubes; which were further subjected to ultrasonic extraction at 20 kHz for 30 min (Vibra cell by Sonics and Materials Inc., USA). Sonicated samples were evaporated to dryness. Residues were collected in 1 ml HPLC grade methanol, centrifuged at 10,621 g for 10 min, at 4 ºC and further stored at -20 ºC for analyses by TLC, HPTLC, HPLC and GC-HRMS techniques.

2.5. Detection and Analysis of CPT

Thin Layer Chromatography (TLC) analysis was carried out to detect CPT content in cell biomass and filtrate qualitatively using TLC silica gel 60 (Merck, Germany) plates. A pure sample of camptothecin (Sigma Chemicals, USA) dissolved in methanol was used as standard and run along with the calli-extracted samples. Solvent system required for chromatographic separation of standard CPT was optimized and optimum separation was obtained using chloroform/ethyl acetate (1:1, v/v) as the mobile phase. Extracts were spotted onto the plate along with standard CPT and developed in a pre-saturated TLC chamber (Camag, Switzerland). The Relative front (R_f value) of standard camptothecin was compared with that obtained in the sample tracks upon observation under UV illuminator (Camag, Switzerland) at 254 nm.

High Pressure Thin Layer Chromatography (HPTLC) analysis was further carried out to confirm the detection and quantification of CPT in the test samples. Samples (10 µl) were loaded on HPTLC silica gel 60 F_{254}s plates (Merck, Germany) along with a series of concentrations of standard CPT (Lower range: 2 ng to 100 ng and higher range: 500 ng to 3000 ng) using Linomat5 applicator (Camag, Switzerland). The plates were developed using the solvent system chloroform/ethyl acetate (1:1, v/v). The chromatographs obtained were air dried and scanned by Camag Scanner3 at 254 nm and the R_f value, peak height and peak area of the spots were obtained using WinCATS software (Camag, Switzerland). Peaks in the sample with R_f value coinciding with that of standard CPT were recorded and further used for estimation of the amount of CPT in the corresponding samples. The CPT content was analysed by integrating the area under the peaks obtained at the same retention time (R_t) as that of the standard CPT (R_t 9.5 ± 0.1 min).

High Performance Liquid Chromatography (HPLC) was performed to quantify the amount of CPT produced by suspension cultures that were generated from different explants. HPLC was performed on DGU-20A 5R (Shimadzu,

Japan) with C-18 column (5 µm x 250 mm x 4.6 mm, Enable, Spincotech Pvt. Ltd., Japan) using mobile phase methanol/water (3:2, v/v) at a flow rate of 0.7 ml/min. The CPT produced by extracted samples was detected using a PDA detector (SPD-M 20 A, Shimadzu, Japan) in dual mode with 1.2 slit width and the chromatograms were extracted at 254 nm. Quantification of the CPT produced was done by spiking the calibration curve with different concentrations of standard CPT (Sigma Aldrich) and the validation was determined by performing 5 replicates of each sample. Data acquisition and post analysis were performed using LC solution software (Shimadzu, Japan).

Gas Chromatography with High Resolution Mass Spectrometry (GC-HRMS) was performed on HP-5MS-UI 30 m × 0.25 mm × 0.25 µm (Agilent-7890 with a FID detector, Agilent Technologies, USA) capillary column. The operating conditions were: 280 ºC for 5 min, solvent delay: 6 min, injection mode: splitless, 0.2 min, injector temperature: 280 ºC and injection volume: 1 µL. Mass spectrometry (MS-Jeol, Model- AccuTOF GCV with EI / CI Source) was performed to confirm the presence of CPT in samples.

3. RESULTS

3.1. Cell Culture

The endospermic tissue showed varying percentages of response to callus induction and proliferation depending upon the growth hormone supplemented in the MS medium (**Table 1**)**.** Explants responded to form cream coloured, soft and friable calli after 4 weeks and were further subcultured onto the same media. Callus formation and proliferation from endospermic tissues was observed over a period of 4-6 weeks with higher frequency on MS basal medium supplemented with 2,4-D (2 mg/L), BA (1 mg/L) and sucrose 20 g/L (**Figure-1[b], [c]**).

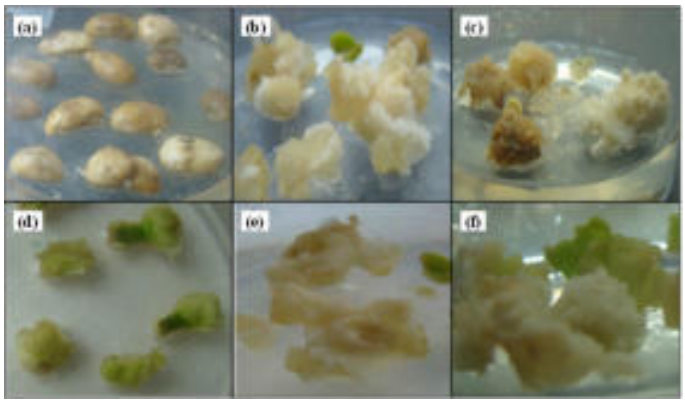

Figure-1: (a) Endospermic tissue of *Nothapodytesnimmoniana* seeds, (b) callus obtained from endospermic tissue, (c) endospermic callus after first subculture, (d) callus obtained from *in vitro* grown stem segments, (e) callus obtained from roots of *in vitro* grown seedlings, (f) callus obtained from *in vitro* grown leaf segments.

Callus was formed on the entire surface of the explants, with friable consistency and varied from creamish-white to brown in colour. Organogenesis was also observed from the calli induced. Upon transfer of the 8-week-old calli to fresh MS medium supplemented with TDZ (2 mg/L), green shoot buds emerged after 2 weeks. These results were in accordance with those reported by [14] wherein TDZ was shown to induce multiple shoot formation in cell cultures of *N. nimmoniana*.

Calli obtained on MS supplemented with NAA (2 mg/L) and BA (0.5 mg/L) showed formation of multiple shoots and adventitious roots upon further incubation for 3-4 weeks (**Figure-2**).

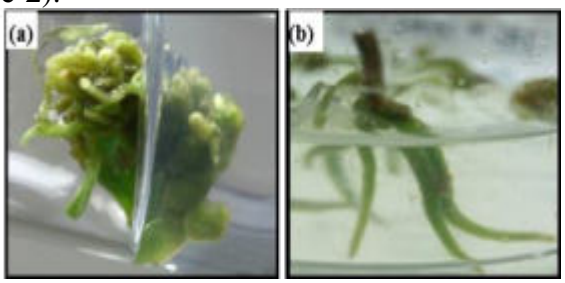

Figure-2: Organogenesis from endosperm derived callus (a) Multiple shoots formation on MS + TDZ (2 mg/L); (b) Root formation on MS + NAA (2 mg/L) + BA (0.5 mg/L).

Few explants did not show any callusing response and turned brown due to exudation of phenolic compounds. To avoid browning phenomenon antioxidant compounds like 0.5 % polyvinylpyrrolidone (PVP) were used in the medium. Callus cultures derived from endospermic tissue showed higher CPT content as determined by HPTLC and HPLC profiles. Suspension cultures were successfully established from 4 week old soft friable calli derived from endosperm tissue on MS medium supplemented with 2,4-D (2 mg/L), BA (1 mg/L) and sucrose 20 g/L. These were further subcultured after every two weeks and maintained on the same medium. Seeds of *N. nimmoniana* germinated within 2 weeks of incubation and further took 2 weeks to grow up to a 5 cm long seedling on MS basal medium. Parts of these plantlets were then used as explants for callus culture using various combinations of PGRs. Callus formation from stem, leaf and root of germinated seedling was observed only in the case of (i) MS supplemented with NAA (2 mg/L) and BA (0.5 mg/L), and (ii) MS supplemented with picloram (2 mg/L, Sigma Chemicals, USA). Callus induction from excised embryos of *N. foetida* using picloram has been reported earlier [10, 15]. Whitish-green coloured, soft and fragile callus was obtained which was further subcultured onto the same medium. But the percentage of obtaining multiple shoots and adventitious roots from the callus formed was lesser and the effect of browning of the explants was more prominent. Browning phenomenon is observed in cultures of woody plants mainly due to exudation of phenolic compounds. Suspension cultures were also developed from calli derived

from *in vitro* grown stem, leaf and root explants on MS medium containing sucrose (20 g/L) and supplemented with the same combination of hormones as mentioned for callus cultures.

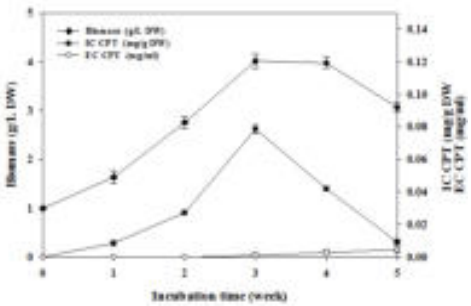

Figure-3: Growth curve and CPT accumulation in cell suspension culture derived from endospermic tissue of *N. nimmoniana* (values are mean of three replicates; error bars are represented as vertical lines).

3.2. Estimation of CPT

Concentration of CPT from endosperm derived callus varied from 0.023-0.98 mg/g dry wt. while cell associated CPT in suspension cultures varied from 0.001-0.079 mg/g dry wt. of biomass (**Table-1**). CPT content in multiple shoots and adventitious roots generated from endospermic calli was negligible and hence not considered in further studies. The highest content of CPT was detected from callus (0.98 mg/g dry wt.) and suspension (0.079 mg/g dry wt.) cultures generated from the endospermic tissues on MS medium supplemented with 2,4-D (2 mg/L), BA (1 mg/L) and sucrose (20 g/L). This content was higher than those detected from calli and their respective suspension cultures generated from stem, leaf and root explants (**Table-2**).

Table-2: Content of CPT in callus and suspension cultures derived from different parts of *N. nimmoniana.*

Sr. No.	Cell suspension culture generated from	CPT [mg/g dry wt. of Biomass] *	
		Callus	Suspension culture
1	Endosperm	0.98 ± 0.016	0.079 ± 0.022
2	Stem	0.42 ± 0.021	0.034 ± 0.017
3	Leaf	0.33 ± 0.028	0.029 ± 0.012
4	Root	Trace amounts	Trace amounts

*Intracellular CPT content detected at the end of 4 weeks of incubation; Values are mean of three replicates ± S.D. as computed using Graph Pad InStat3 software (La Jolla, CA, USA).

Calli and suspension cultures generated from root explants produced only trace amounts of CPT. CPT content was also computed by HPTLC technique using calibration curve method (**Figure-4**) and the results obtained were comparable to the values obtained by HPLC technique.

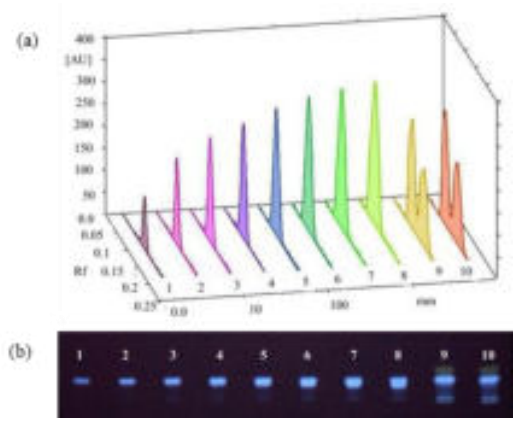

Figure-4. HPTLC chromatogram of (a) calibration curve track no. 1 to 8 correspond to increasing conc. of standard CPT; track no. 9,10 are methanolic extract of biomass from endospermic cell suspension culture (b) HPTLC silica gel image of the same.

Also, the average percent response of callus formation from the endospermic tissues (78.6%) was higher as compared to that of callus formation from leaf (68.1%), stem (72%) and root (54.34%) explants within a period of 4-6 weeks. The period of incubation for optimum growth of endospermic callus was found to be 4 weeks and further subcultured on the same medium for 3 weeks. Maximum concentration of CPT from suspension cultures was obtained during 3-4 weeks of incubation after which it declined. Similar results were obtained in case of cultures of second and third subculture on the same medium containing 2, 4-D (2 mg/L), BA (1 mg/L) and sucrose (20 g/L). The level of CPT decreased after a 3 weeks period due to lack of nutrients. GC-HRMS analysis confirmed the presence of CPT in the extracts as detected against the chromatogram of standard CPT (Retention time R_t 11.76 ± 0.2 min). The fragmentation patterns obtained in case of the standard CPT compound and the methanolic extract of endosperm-derived cell cultures were comparable (**Figure-5**) [16]. These results signify the use of high yielding endospermic tissues for CPT production from callus and cell suspension cultures on shake flask scale.

4. DISCUSSION

Ultrasound assisted extraction (UAE) method was used to extract CPT from the cultures. UAE facilitates mechanical shearing forces due to acoustic cavitations that disrupt plant cell walls more efficiently and improve release of cellular extracts into

the solvent [17]. Sonication method has been considered as an efficient, simple, cost effective and lesser time-consuming extraction method over traditional methods of cell disruption like stirring, soxhlet and maceration methods [18]. Earlier studies on CPT content in cell suspension cultures derived from various explants of *N. nimmoniana* have shown significantly lesser CPT production than that in the wild type explants [10,11]. These results suggest that dedifferentiation (callus formation) leads to decrease in the content of CPT and hence low productivity from suspension cultures too. Trace amounts of CPT detected in root-derived callus can be correlated to these earlier studies. The content of CPT in root explants of the plant is comparatively lesser (1.87 mg/g dry wt. of roots) as reported earlier [19].

Endospermic tissues showed CPT up to 7.6 mg/g dry wt. of explant. The high content of endogenous CPT in the endospermic tissue probably accounts for the higher content of CPT in the endosperm-derived cell cultures. Accumulation patterns of CPT have been observed to vary with the plant parts *viz.* leaves, stem and seeds (embryo and endosperm) in *Camptotheca accuminata* [20]. Similar distribution of the alkaloid is observed in *N. nimmoniana* too with significant amounts in the endospermic tissues of the seeds [21]. It has been hypothesized that CPT tends to accumulate rather than getting synthesized in the seed and so must be in the endosperm [22]. Significantly high amounts of CPT (0.93% dry wt.) produced by callus generated from immature cotyledons have been reported [23]. Biomass associated CPT accumulation with maximum intracellular CPT (0.0298 mg/g dry wt. of cell biomass) has been detected and reported in suspension cultures derived from leaf explants of *N. nimmoniana* [24]. According to their findings, CPT accumulation was associated with biomass production and trace amounts of CPT were leached out in the medium after the onset of stationary phase. We also checked extracellular (filtrate) CPT content after every week and only trace amounts could be detected only at the end of 4^{th} and 5^{th} weeks of incubation. Maximum biomass (4.02 g/L dry wt.) and intracellular CPT (0.079 mg/g dry wt.) production was detected after 3 weeks of incubation; after which both the parameters showed noticeable decrease (Figure-3). Extracellular CPT production was negligible at the end of 3 weeks and was detectable in trace amounts only till end of 4^{th} week. These studies were contrary to those reported earlier [25] wherein extracellular CPT was significant to intracellular CPT in *N. nimmoniana* suspension cultures. The noticeable amount of CPT produced by cell suspension cultures developed from endospermic tissues reported in the present study paves the way for further optimization studies and large-scale production of the potent anti-cancer drug derivative.

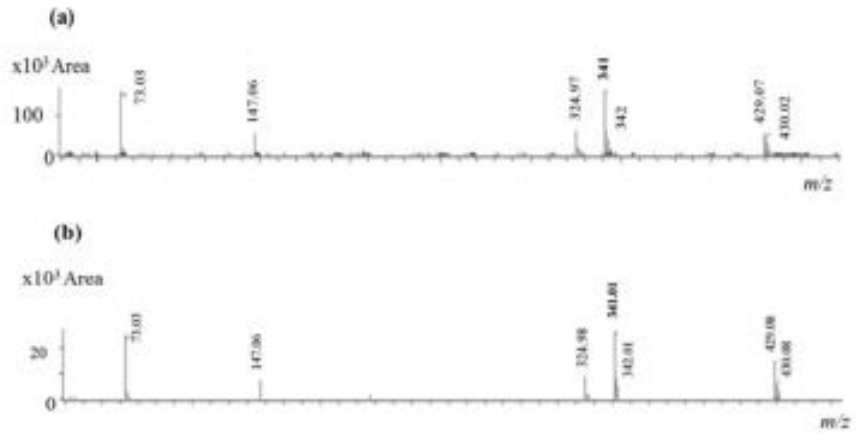

Figure-5. High Resolution Mass Spectrometry pattern of (a) Standard CPT with a signal at *m/z* 341 and (b) Methanolic extract of biomass from endospermic cell suspension culture.

5. CONCLUSION

The present study aimed to use endospermic tissues of *N. nimmoniana* for induction of callus and suspension cultures with higher CPT accumulation. The study implies application of endospermic tissue derived calli and suspension cultures for commercial production of CPT following yield improvement methods so as to meet the ever-increasing market demand for the potential anti-cancer drug. The study also suggests that no further differentiation of the callus is required for obtaining high yielding suspension cultures, conversely as in case of cell cultures derived from leaf and stem segments. Endospermic tissue is easily available from *N. nimmoniana* seeds and this practice will definitely aid in conservation of the endangered species. Seeds can be freeze-stored and used for clonal propagation and thereby CPT production applying biotechnological strategies. Future perspectives of the present study include large scale propagation of the endospermic calli and suspension cultures. Production of CPT from cell cultures and its application for medicinal use shall be studied extensively.

ACKNOWLEDGEMENTS

Bhumika N Bhalkar wishes to acknowledge Council of Scientific and Industrial Research (CSIR), New Delhi, India for the award of Junior Research Fellowship as well as Indian Institute of Technology – Sophisticated Analytical Instrument Facility (IIT-SAIF), Mumbai, India for facilitating GC-HRMS analysis of samples. Prof. Sanjay P Govindwar is thankful to the Department of Biotechnology, New Delhi,

India for funding the Interdisciplinary Program for Life Sciences, education and research (IPLS) program to Shivaji University, Kolhapur, India.

AUTHOR CONTRIBUTION

Bhumika N Bhalkar has designed and executed the experimental research work represented in the publication as well as drafted the manuscript. Prof. Jyoti P Jadhav has guided throughout the experimental phase and scientifically corrected the manuscript.

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Green synthesis of gold nanoparticles using *Mucuna pruriens* var. *utilis* with its biomedical potential

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ABSTRACT
The development of ecologically safe and reliable metallic nanoparticles is a significant measure in nanotechnology. To accomplish this use of natural sources, such as biological systems become indispensable. Synthesis of gold nanoparticles using Mucuna pruriens var. utilis seed extract has been investigated in this study. The nanoparticles were produced quickly by optimizing experimental variable parameters such as a 0.5:1mM ratio of plant seed extract to gold chloride, a temperature of 65°C, and a pH of 9. The morphology and crystalline phase of nanoparticles were examined using UV visible spectroscopy, energy dispersive X-ray (EDS) spectroscopy, Fourier transform infra-red (FTIR) spectroscopy, and X-ray powder diffraction (XRD) pattern analysis. The UV-visible spectroscopy of synthesized gold colloidal solution exhibited absorption maxima at 538 nm. The AuNPs showed excellent dose-dependent antioxidant activity using FRAP assay moreover AuNPs showed good anti-inflammatory activity using the membrane stabilization method. Vero cells were used to test cytotoxicity since they showed cell viability at greater concentrations.

KEYWORDS
Anti-inflammatory activity, Cytotoxicity, Gold nanoparticles, FTIR, FRAP assay.

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ABBREVIATION

AuNPs-gold nanoparticles,BBB- Blood brain barrier, EDS- Energy-dispersive X-ray spectroscopy, FRAP- Ferric reducing antioxidant power, FTIR-Fourier transform infrared spectroscopy, $HAuCl_4.4H_2O$- Gold chloride/ chloroauric acid, MTT-3-(4,5-diamethylthiahiazol-2yl)-2,5-diphynyl tetrazolium,RT- Reaction time, TEM- Transmission electron microscopy, TPTZ-2,4,6-Tripyridyl-s-triazine, XRD-X-ray diffraction analysis.

1. INTRODUCTION

Nanotechnology is the study of nanoscale materials with diameters ranging from 1-100 nm and their applications [1]. The term 'nano' is derived from the Greek word 'dwarf' which means small in size. It means 10^{-9} or 0.000000001 when used as a prefix. One billionth of a meter is one nanometer (nm) [2]. Because of their huge surface area and electrical characteristics, nanoparticles have distinct physical and chemical properties in comparison with solid bulk materials. These particles have also been used for a wide range of applications, such as electrochemistry, photochemistry, and biomedicine [3]. Gold nanoparticles are used in a range of biomedical applications, diagnostic as well as in drug delivery [4].

Despite the popularity and advancement of chemical and physical methods for producing nanoparticles, the need to develop environmentally acceptable methods that do not involve the use of harmful chemicals is critical, particularly for medicinal purposes [5]. Synthesis methods that use natural products as reducing agents require extra attention to reduce environmental and health risks. Plant-mediated synthesis, on the other hand, is advancing due to the simplicity with which the size and form of nanoparticles may be controlled, as opposed to the challenges encountered in microbe-assisted synthesis [6]. Plant-based synthesis is quick, safe, and easy to do, and it can be done in a room without requiring a lot of energy.

Gold nanoparticles are frequently used in biotechnology and biomedicine because of their excellent electron conductivity and vast surface area [7]. Gold nanoparticles were found to be the safest and least hazardous drug delivery agents [8]. *Mucuna pruriens* var. *utilis* Wall. ex-Wight (Fabaceae) is an underutilized legume found in Southern and Southeastern Asian regions [9]. The velvet bean is high in protein (23-35%) and nutritionally comparable to other pulses like rice bean, soybean, and lima bean [10]. They are the source of valuable phytocompounds, as well as nutritional substances [11]. *Mucuna* species are being utilized for the management of Parkinson's disease [12] with its promising content of Levodopa (~5–11%) moreover *Mucuna* has potent anti-oxidant activity, iron chelating, and anti-inflammatory potential [13]. The current study investigates plant-mediated optimized biosynthesis of AuNPs using methanolic *M. pruriens* var. *utilis* seed extract. UV-visible and FT-IR spectroscopy, XRD, EDS, and TEM investigations were used to characterize and validate the optimized gold nanoparticles. The FRAP method was used to determine the antioxidant activity of produced AuNPs, and the membrane stabilization method was used to determine the anti-inflammatory activity. The cytotoxicity of the purified biogenic AuNPs was assessed against Vero cell lines using MTT assay.

2. MATERIALS AND METHODS

2.1. Materials

Chloroauric acid ($HAuCl_4.4H_2O$), 2,4,6-Tripyridyl-S-triazine (TPTZ), and Ferric chloride ($FeCl_3$) were procured from Hi-Media, India. *M. pruriens* var. *utilis* was collected from Nanded, Maharashtra. The voucher no. SVG009 has been deposited at the Department of Botany, Shivaji University, Kolhapur (SUK).

2.2. Preparation of plant extract

The seeds were dried under sunlight for 5 days, powdered into a fine powder, and kept at room temperature until they were used. One g of *M. pruriens* var. *utilis* seed powder was added with 100 ml of methanol and remains on a rotator shaker for 1 h. further sonicated for 15 min. The extract was sieved through Whatman no. 1 filter paper and kept at 4ºC for further use.

2.3. Optimization of biosynthesis of AuNPs

Study of different physicochemical parameters such as metal ion concentration $HAuCl_4.4H_2O$, a proportion of seed extract to $HAuCl_4.4H_2O$, temperature, pH, and reaction time (RT) may affect the synthesis of the nanoparticles. For this, the effect of metal ion ($HAuCl_4.4H_2O$) in the range 0.25-1.5 mM; the ratio of seed extract to $HAuCl_4.4H_2O$ in the range 0.1:1-0.5:1 (v/v); pH at 3,5,7,9 and 11 respectively; temperature in the range of 35ºC, 45ºC, 55ºC, 65ºC and 75ºC, and the reaction mixture was observed at different time intervals (0 h, 2-6 h, 12 h and 24 h). A change in the color of the reaction mixture from yellow to purple-red marked the synthesis of AuNPs. On a Shimadzu spectrophotometer, the absorbance of the reaction mixture was measured by recording the UV-visible spectrum between 400 and 800 nm. (Model UV- 1800). To achieve the pellet, the colloidal solution containing AuNPs was centrifuged at 15,000 rpm for 15 min. at room temperature after optimizing the reaction conditions. To minimize any biological impurities, the pellet was thoroughly washed with deionized water at least 3-4 times. The particles were dried in the air until being kept for future use.

2.4. Characterization of AuNPs

Various spectroscopic and imaging techniques were used to validate the synthesized AuNPs. A UV-visible double beam spectrophotometer was used to record the reaction mixture's UV-visible spectra from 400 to 800 nm with a resolution of 1nm. (Shimadzu). Blank was adjusted with $HAuCl_4.4H_2O$, and over a while, the stability of AuNPs was observed.Fourier transform infrared spectroscopy (FT-IR) was used to study the functional groups of *Mucuna* extract and purified AuNPs in the range of 500-4000 cm^{-1}on an FTIR spectrophotometer (Shimadzu, Japan). By placing a colloidal solution drop of purified nanoparticle solution on a JEOL (JEM 2100F) operated at 200 kV with resolution 2.4 A, the size and morphology of the

nanoparticles were imaged. To identify the elemental compositions, EDS with x-act with INCA and Aztec EDS analysis software (Oxford Instruments, UK) was used to detect the functional group. By recording the XRD pattern on Bruker AXS Analytical Instruments, the crystalline nature of purified AuNPs was analyzed using X-ray diffraction (XRD) pattern analysis (Bruker D2 phaser Pvt. Lt., Germany).

2.5. Assessment of antioxidant potential by FRAP assay

Benzie and Srrain(1996) [14] protocol was followed to assess the antioxidant potential of AuNPs. Briefly, the working FRAP reagent was prepared by mixing 300 mM Na-acetate buffer (pH 3.6), 10 mM (TPTZ) in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio before use and heat to 37°C in a hot water bath for 10 min. With 3.0 ml of FRAP reagent, the various concentration of synthesized AuNPs (100-500µg/ml) were allowed to react. With distilled water, the reaction mixture was brought up to a final volume of 4.0 ml. For 30 min., the reaction mixture was held in the dark at room temperature. Using a UV-visible spectrophotometer, the absorbance of the colorful product (ferrous tripyridyltriazine complex) was measured at 593 nm. A greater reducing power was indicated by a higher absorbance reading.

2.6. *In vitro* anti-inflammatory activity by membrane stabilization test

Fresh human blood was drawn and mixed with an equivalent volume of saline solution (pH 7.2, 0.85 %). The packed cells were rinsed three times with saline [0.85%, 7.2pH] afterward centrifugation at 3000 rpm for 10 min. The packed blood cell volume was determined and reconstituted as a 10% v/v saline suspension [15].

The heat-induced hemolysis experiment was carried out [16, 17]. The reaction combination (2 ml) contained 1 ml of a test sample of different concentrations (100-500 µg/ml) of AuNPs and 10% RBCs suspension (1 ml); the control test tube contained only saline instead of a test sample. Aspirin was used as a routine medication. For 30 min., all of the centrifuge tubes containing the reaction mixture were placed in a water bath at 56°C. The tubes were cooled under running tap water at the end of the incubation. The reaction mixture was centrifuged for 5 min. at 2500 rpm, and the absorbance of supernatants was measured at 560 nm. For all of the test samples, the experiment was repeated three times.

$$\%\ \text{inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

2.7. Cytotoxicity assay

Vero (African green monkey kidney) cell line was employed to study the cytotoxicity. Cells were cultured in DMEM-Sigma complemented with 10%fetal bovine serum (Sigma) and penicillin-streptomycin antibiotic solution (Sigma). 1 X 10^6Vero cells were seeded into 96-well plates and placed in a humidified atmosphere containing a 5% CO_2 incubator at 37 °C and allowed to grow to 90% of confluence.

After a 24 h incubation period, adherent cells were treated with different concentrations of synthesized AuNPs (50-250 µg) and re-incubated for 24 h. Further, the added media was removed and washed by PBS-pH 7.4and replaced with 40 µL MTT (1 mg/mL). The plate was incubated for 4 h. The media containing MTT solution was removed, and synthesized formazan crystals were solubilized by adding 100 µl of DMSO. The absorbance was measured in an ELISA reader (Thermo Scientific Multiskan Sky) at 570 nm. The percentage of cell viability was calculated as:

$$\% \text{ Viability} = \frac{(\text{absorbance of treated cells} - \text{absorbance of blank})}{(\text{absorbance of control} - \text{absorbance of blank})} \times 100$$

3. RESULTS AND DISCUSSION

3.1. Biosynthesis of AuNPs

In the current study, *M. pruriens* var. *utilis* seed extract was used as a reducing and capping agent in the biogenic synthesis of AuNPs. The addition of gold chloride changed the color of the *Mucuna* seed extract to dark purple, confirming the synthesis of gold nanoparticles. The color change was due to the existence of different phyto-compounds in the plant extract as it reduces Au^{3+}ionsto Au^{0} (Sunderam et al., 2019) [18]. UV–visible spectroscopy and TEM studies were also used to optimize reaction conditions such as the amount of *Mucuna* extract, temperature, gold chloride ratio, pH, temperature, and reaction time.

3.2. Optimization of biosynthesis AuNPs

For optimizing the biogenic synthesis of AuNPs, several parameters such as the effect of pH, temperature, the various concentrations of $HAuCl_4 \cdot 4H_2O$, and the ratio of plant extract to $HAuCl_4 \cdot 4H_2O$ were varied and examined. The UV–visible spectrophotometer was used during the studies to display the stability and development of nanoparticles in methanolic solutions. In the visible range, AuNPs showed a distinctive SPR peak between 500 and 570 nm, indicating that they had been synthesized. The absorption spectra of AuNPs formed at varying concentrations of $HAuCl_4 \cdot 4H_2O$ (0.25mM-1.5mM) are shown in Fig.1a. The 1mM concentration was detected to signify the maximum SPR peak. The greatest SPR peak was recorded at a concentration of 1 mM. There was an elevated peak of up to 1mM concentration with a small shift of the SPR band towards a longer wavelength as the concentration was gradually increased. There was a decline in peak intensity at higher concentrations as the SPR band moved towards the longer wavelength area **(Figure-1a)**. The change in the SPR band in the current study may be due

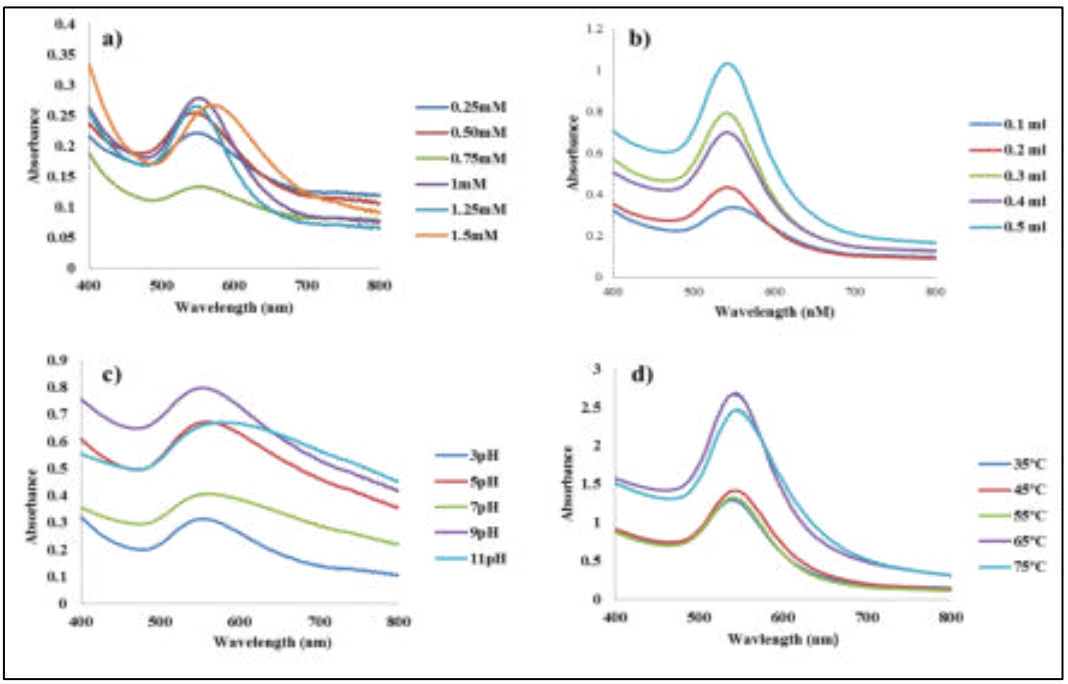

Figure-1. UV-visible absorption spectra of optimized biogenic synthesis of AuNPs. a) Effect of varying concentration of $HAuCl_4$; b) effects of various ratio of seed extract; c) effect of varying pH; d) effect of incubation temperature.

to the larger size of AuNPs. At a constant concentration of $HAuCl_4 \cdot 4H_2O$, the effect of various volumes of seed extract (0.1-0.5ml) was studied. The optimum ratio for synthesizing AuNPs with maximal SPR peak intensity was found to be0.5:1. The increased volume of plant extract resulted in a drop in peak intensity **(Figure-1b)**. The synthesis was confirmed in the visible region by a peak and was visible by the color change from yellow to purple caused by SPR vibration excitations.

The pH of the solution is also a significant factor in the reduction, synthesis, and stabilization of AuNPs. During synthesis, Au^{3+} interacts with the pH-dependent functional groups found in plant extracts. The oxidation state and the power of secondary metabolites are both affected by pH [19]. The synthesis of nanoparticles for pH range 3-11was investigated in the present study. The synthesis was studied by a UV–visible spectrophotometer and by a color shift for all pH values. Here, SPR peak intensity was elevated as the pH was increased up to 9 pH, presenting optimum for the AuNPs synthesis. At pH 11, peak intensity was lower in comparison with pH 9 **(Figure-1c)**. Peak broadening was detected at lower pH a value, reflecting the synthesis of larger AuNPs, but peak broadening was reduced at optimum pH, representing the synthesis of smaller AuNPs.

The temperature of the reaction has also been proved to be an indicator to determine the rate of nucleation and reaction, yield, growth, and other characteristics of AuNPs using these parameters [20]. There was an increasing reduction of gold salt with a small blue shift as the temperature is elevated. Shifting to the blue wavelength indicates that the size of the nanoparticles reduces as the temperature rises, owing to the faster reaction rate at higher temperature. As the kinetic energy of the molecule increases, these gold ions are absorbed more rapidly at higher temperatures [21]. The SPR peak intensity was improved steadily at varied incubation temperatures ranging from 35°C to 75°C, with the highest peak intensity recorded at 65°C (**Figure-1d**). The rate of AuNP synthesis was enhanced to 65°C, indicating that the rate was temperature-dependent. Furthermore, it was revealed that the SPR peak intensity declined when the temperature rose above 65°C.

3.3. Characterization of AuNPs

AuNPs were confirmed by the appearance of a peak in the visible region at 538 nm and a colour change to purple. Rapid production of biogenic nanoparticles was followed by a 7-day stability assessment after providing appropriate circumstances (**Figure-2a**). FTIR spectral analysis has been evaluated to investigate the functional groups responsible for capping or reducing agents in this method of nanoparticle synthesis. The FT-IR spectra prepared extract; synthesized AuNPs were recorded at room temperature. The broad stretching has been observed at 3321.62cm^{-1}, and 3323.09 cm^{-1} attributed to (O–H) alcohol and phenol group in plant extract and synthesized AuNPs respectively. The stretching band observed at 2941.70 $cm^{-1}$and 2830.24 cm^{-1} correspond to (O–H) in carboxylic acid and (C–H) in aldehydes in plant seed extract, whereas, there is an absence of the same functional groups in synthesized nanoparticles. The stretching has been recorded at1700.07 $cm^{-1}$and 1640.05 cm^{-1} **(Figure-2b)** showing the presence of (-C=O) carbonyl group in plant extract and synthesized AuNPs, which is prominent in the synthesized AuNPs. Also, stretching vibrations at 1536.10 $cm^{-1}$and 1512.46 cm^{-1}attributed to N–O symmetric stretch which leads to the presence of nitro compounds in plant seed extract, whereas the same stretching was absent in synthesized AuNPs. Another stretching vibration has been marked at 1458.83cm^{-1}, 1407.56$cm^{-1}$for plant extract, and 1462.31$cm^{-1}$for synthesized nanoparticles accrediting to the (C–H) group of alkanes. The stretching band observed at 1113.40 attributed to (C–N) stretch of aliphatic amines in the prepared plant extract. The prominent stretching has been observed at 1020.38 $cm^{-1}$for plant extract, whereas narrow stretching was observed at 1014.72 cm^{-1} in synthesized AuNPs leading to (C–O) stretching of alcohols, carboxylic acids, esters, and ethers. The medium stretching vibration has been recorded at 636.57 cm^{-1}shows the presence of alkynes in the plant extract. Evaluating FTIR spectra of both plant extract and synthesized AuNPs indicated vibrational stretching of (-C=O), (C–O),

(O–H), (N–O), (C–N), implying that the functional group of esters, alcohol, carboxylic acids, phenol, and ethers found in *M. pruriens* var. *utilis* seed extract mediates the mechanism of capping and bio-reduction in AuNPs synthesis (**Figure-2b**).

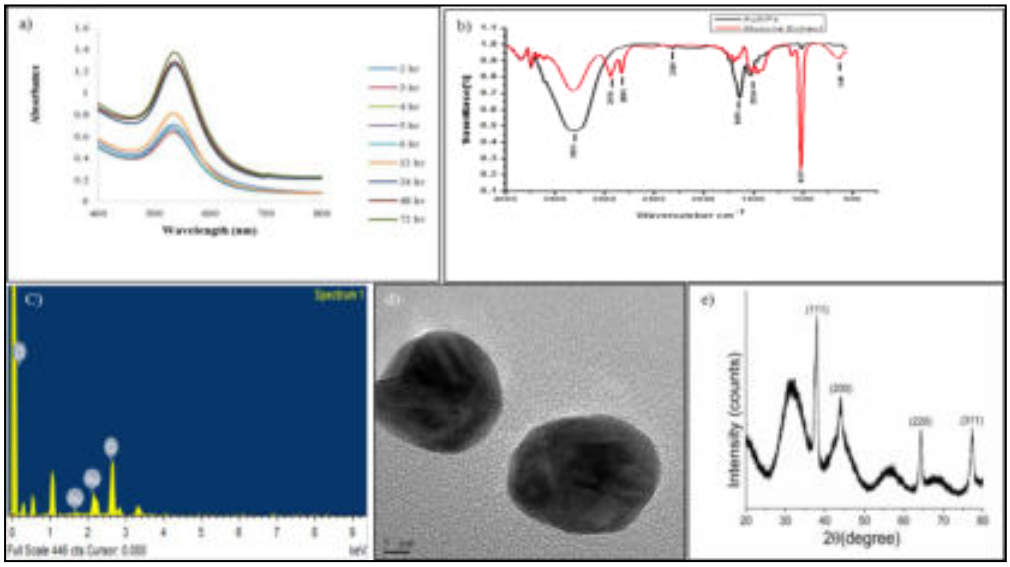

Figure-2. Characterization of biogenic AuNPs. a) Effect of incubation temperature of purified AuNPs; b) FT-IR spectra of purified AuNPs and *Mucuna* extract ; c) energy dispersive X-ray (EDS) spectra of AuNPs presenting peaks of gold, oxygen and carbon; d) transmission electron microscope image showing particle size of AuNPs at different scale 5–10 nm; e) X-ray diffraction (XRD) purified AuNPs.

SEM with an EDS detector was used to determine the elemental composition of powdered samples. **Figure-2c** shows the results of an energy dispersive X-ray study (EDS), which exhibited a high signal in the gold region and validated the creation of AuNPs. Due to surface plasmon resonance, metallic Au nanocrystals have a typical optical absorption peak of about 2.2 keV. The form and size of biogenic AuNPs were examined using TEM. AuNPs with an average size of 5nm were found to be spherical using the TEM technique (**Figure-2d**). **Figure-2e** shows the X-ray diffraction profile of Ag NPs. The XRD pattern of AuNPs was recorded and analyzed for the investigation of crystalline nature and revealed four intense peaks of 2 values in the range of 10-90. These four unique diffraction peaks at 36.58°, 42.71°, 61.90°, and 74.78° matched to crystallographic planes (111), (200), (220), and (311). The Bragg reflections perfectly linked the reported reference values of Joint Committee on Power Diffraction Standards (JCPDS) card No. 03–0921,

demonstrating that AuNPs were composed of pure crystalline Au and resembled the face-centered cubic (FCC) structure of metallic Au.

3.4. Antioxidant activity

Antioxidant compounds are stable molecules that give an electron to a free radical, neutralizing it and minimizing the vulnerability of oxidative damage [22].The imbalance between reactive free radicals and the antioxidant defense system causes oxidative stress, which leads to a variety of chronic diseases by causing cellular deterioration and, eventually, death [23]. One of the factors that cause neurological diseases, aging, and diabetes is oxidative stress. Many drugs can neutralize the free radicals but are incapable to cross the BBB; hence plant-based drugs on nanoparticles are of particular relevance. As a result, these phytcompounds are the agents responsible for the decrease of Au while also having antioxidant capabilities.

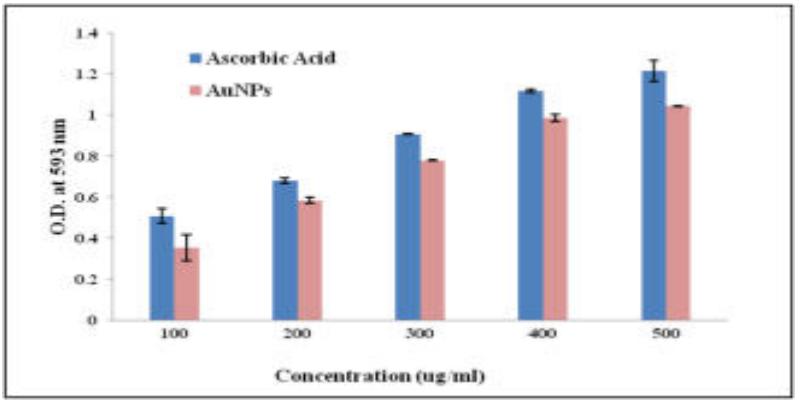

Figure-3. FRAP radical scavenging activity of purified AuNPs.

To elucidate antioxidant potential of *M. pruriens* var. *utilis* AuNPs FRAP scavenging activity was investigated. FRAP activity was used to evaluate antioxidant ability by donating electrons to free radicals to convert ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+})[24].The FRAP scavenging activity of synthesized AuNPs is shown in **figure-3**. The scavenging ability at 300µg/ml concentration of AuNPs was found to be 0.76 ±0.05 absorbance in comparison with 0.91 ± 0.02 absorbance at the same concentration of reference standard ascorbic acid.

3.5. Anti-inflammatory activity

Inflammation is the immune system's response to stimuli due to tissue damage, and it is a significant natural defense mechanism for healthy life. Membrane stabilization is the process by which anti-inflammatory drugs maintain the integrity of the erythrocyte membrane and lysosomal membrane by stabilizing the membrane. This stabilizing effectof anti-inflammatory drug on the erythrocyte membrane may be due

to the drugs' stabilizing effect on certain proteins in the membrane [25]. .HRBC membrane stabilization assay indicated that the synthesized AuNPs were effective in inhibiting heat-induced hemolysis and showed a concentration-dependant increase in % membrane stabilization (**Table-1**). The AuNPs showed maximum inhibition of 39.49 ± 0.57% at a concentration of200µg/ml, whereas Aspirin a standard anti-inflammatory drug showed 42.5 ± 0.8% at that concentration. Similarly, at 500µg of aspirin, the membrane stabilization was 60.73 ± 0.7%, and at the same concentration, AuNPs showed 58.79 ± 0.58%.

Table-1. Anti-inflammatory activity of purified AuNPs with Std. Aspirin.

Concentration (µg/ml)	**Aspirin (%)**	**AuNPs (%)**
100	38.54±0.5	35.4±0.03
200	42.5±0.8	39.49±0.57
300	49.4±0.12	45.63±0.78
400	55.5±0.69	51.07±0.69
500	60.73±0.7	58.79±0.58

Values are expressed as mean±SD

3.6. Assessment of the cytotoxic effect of synthesized AuNPs

Because of their cytocompatibility, stability, and binding capability, gold nanoparticles are an excellent nanocarrier of anti-cancer medications in the field of nanomedicine [26]. In preclinical investigations, in vitro toxicity evaluations are used as a substitute for the assessment of animal toxicity. The cytotoxicity of produced seed extract mediated AuNPs was tested on Vero cell lines using the MTT method. Cell viability was reduced in a concentration-dependent manner when the cells were treated with AuNPs. The cell viability of various doses is depicted in **figure-4**, and IC_{50} was calculated. The cell viability was found to be 86.52% at a concentration of 100µg/ml AuNPs, and 42.37% at a concentration of 500µg/ml AuNPs, with an IC_{50} of 422.47µg/ ml. Similarly, gold particles synthesized from *P. parvum* bulb extract executed IC_{50}424.17 µg/ ml tested on Vero cell lines executing similar results [27]. As the concentration was enhanced, the viability of cells gradually declined. As a result, it suggests that AuNPs are safe to use at a concentration below 422.47 µg/ml and have no cytotoxic effect. The results showed that the AuNPs had higher cell viability at 100µg/ml than the other concentrations investigated.

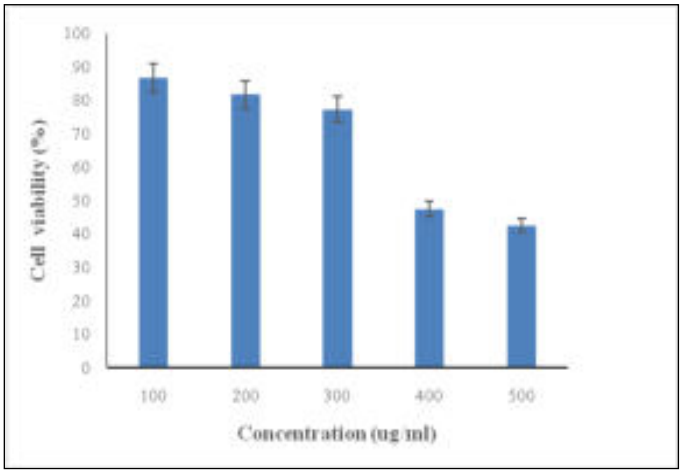

Figure-4. Cytotoxicity of purified AuNPs against Vero cell lines.

4. CONCLUSION

The present study demonstrates the use of *M. pruriens* var. *utilis* seed extract for an easy and convenient approach to the production of AuNPs. The synthesis of experimental variables such as 1mM $HAuCl_4.4H_2O$, a 1:0.5 ratio of $HAuCl_4.4H_2O$ to extract, temperature 65ºC, and pH 9.0 was found to be optimal for biogenic synthesis. The synthesized AuNPs showed excellent antioxidant and anti-inflammatory activity with less toxicity on Vero cell lines depicting safe use of synthesized particles. There is the possibility of L-DOPA coating to the particles during the synthesis process and thus can be applicable for the Parkinson's treatment in future.

ACKNOWLEDGMENT

Manali R. Rane gratefully acknowledges the Department of Biotechnology, Shivaji University, Kolhapur for providing the Golden Jubilee research Fellowship (GJRF). Devashree N. Patil wishes to thank DST-INSPIRE's meritorious fellowship from DST, New Delhi, India.

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Enzymes: In decolorization and degradation of the textile dyes

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ABSTRACT

Presently, the textile and dyestuff industries wastewater treatment by enzymatic degradation exhibited more attention. This was due to high enzymes substrate specificity and comparatively easy to manipulate in the industrial processes. Different enzymes like, azoreductase, chromate reductase, cytochrome P450 and laccase are primarily involved in biotransformation of different types of dyes with various mechanisms in bacteria, fungi and plants. The oxidoreductases have been found easy to harvest, immobilized, and engineered and can be applied in the textile wastewater treatment. The present review explains the mechanism of various enzymes involved and its advances in dye biotransformation which leads to an effective option in the textile wastewater management.

KEYWORDS

Biotransformation, Catalysis, Enzyme, Textile dye.

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1. INTRODUCTION

The chemical synthesizing industries are important for economic development of any country. However, increasing worldwide industrial development resulted into releases large volumes of potentially toxic and xenobiotic compounds like, halogenated, polycyclic aromatic hydrocarbons, dyes, surfactants, radioactive materials, and heavy metals into the ecosystem. Among these, dyes and dyestuff represent one of the main pollutants that are frequently used in the textile, printing, paper, pharmaceutical, cosmetics, food, photographic industries, etc. [1,2]. Nevertheless, among these, textile industry consumes around 80% of the dyes in total production [3,4]. Out of this, about 10-50% of the dyes and dye-stuffs used in different processes are released into effluent treatment systems or directly into the surrounding environment [5]. As the textile industry is one of the key consumers of water and hence is high water polluters in the world. Also, they consequently release huge volumes of effluent having intense color, high organic, inorganic load like Cr, As, Cu, Co, Zn, etc., with extreme temperature and pH fluctuations. The co-existence of such toxicant in wastewater causes serious environmental deterioration. More than

100,000 different dyes have been generated worldwide every year comprising total 280,000 tons are released in industrial wastewater [6,7]. Depending on the chromophores, dyes can be classified into various classes. This includes acridine, azo, arylmethane, anthraquinone, nitro, xanthenes, quinine-amine dyes, etc. [8]. Globally increasing demand for synthetic dyes led to remarkable expansion of dyes industries.

1.1. Impact of textile dyes

The presence of dyes along with other contaminants leads to inhibition of photosynthetic, and oxygenation process leading to high values of biological and chemical oxygen demand (BOD and COD). Many dyes generate toxic, carcinogenic/mutagenic chemicals viz. phenol, benzidine, naphthalene, different aromatic amines which affect the aquatic system mainly due to low light penetration along with oxygen depletion and subsequently affecting entire ecosystem [9,10]. The effluents having low BOD_5/COD ratio (<0.1), which represents recalcitrant nature of dyes for biodegradation [11]. Among the different dyes, as per their chromophoric groups, azo, anthraquinone, triarylmethane and thalocyanine are the major dyes. Out of these different dyes, azo dyes accounts for 70% of textile dyestuffs produced followed by anthraquinone series of dyes [9]. The increase in risk of cancer in people who worked in dyestuff production has been reported previously mainly due to the use of certain aromatic amines for dye production. Literature studies showed that exposure of dyes may cause splenic sarcomas, bladder cancer, hepato-carcinomas and nuclear anomalies, chromosomal aberrations in experimental animals and mammalian cells [7,10]. In consideration with the environmental influences of dyes and its co-contaminants, it is essential to detect and assess proficient decolorization and detoxification method used for dyes before to its disposal into the surrounding environment.

The wastewater generated from dye manufacture and textile industry not only contains color but also has high COD, BOD, total organic carbon (TOC) as well as suspended and dissolved solids. Various physical, chemical, electrical, biological and combination of treatment methods have been used to treat the effluent in an efficient way.

1.2. Biological treatments for textile dye

Potential of biological methods by using bacteria, fungi, yeast, and algae have been well documented for decolorized and/or mineralized dye with involvement of various microbial cultures by enzymatically. Compared with physical and chemical methods, these methods are most economical alternative to treat the textile effluent. Various bacterial species from the genus viz., *Paenochrobactrum, Bacillus, Lysinibacillus, Aeromonas, Alcaligenes, Citrobacter* and *Stenotrophomonas* were recognized as potent candidates for biotransformation of various dyes, Disperse red 167, Acid red 1

and 119, Orange M2R, Reactive blue 160, Reactive blue 4, Reactive red 2, and Direct red 121 with the involvement of different enzymes [7,12].

1.3. Enzymes involved in dye degradation

Currently, the enzymatic technology is gaining more attention in dyes wastewater treatment, due to its substrate specificity and capable to engineer [13]. The involvement of diverse enzymes such as oxido-reductase enzymes viz., azoreductase, DCIP-reductase, cytochrome P450, nitrate reductase, polyphenol oxidase and peroxidases (lignin and manganese peroxidases) and laccases have been largely targeted for biotransformation of dyes.

1.3.1. Oxidoreducatse

Reductase is the most important subclass of oxido-reductase which catalyzes transformation of various organic and inorganic contaminants like dyes, polyphenols, chromate, nitrate, etc. Wastewater from textile and dyestuff industries is difficult to treat, because of a variety of complex structures like dyes having azo, aromatic and heterocyclic groups with presence of surfactants, heavy metals and salts.

1.3.1.1. Azoreductase

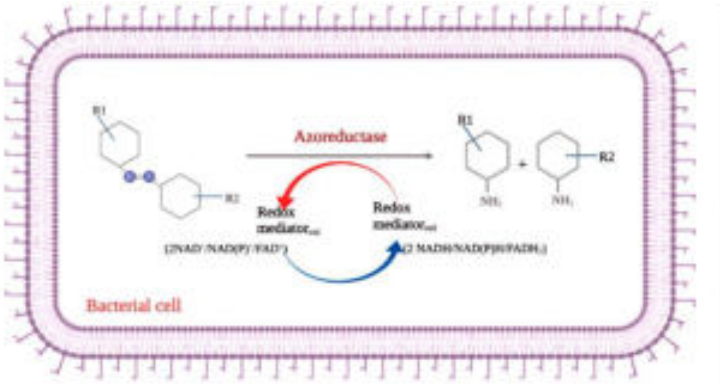
Azoreductase (EC 1.7.1.6) are the significant group of enzymes involved in azo dye decolorization/degradation predominantly expressed in bacteria and fungi. Though, it has diverse structure and function, mostly it decolorized the azo dye by breakdown of azo bond to its corresponding amines [14,12]. Azoreductase enzyme is classified in two groups as a cytoplasmic or membrane bound reductase which greatly flavin dependent or flavin independent. Flavin dependents are further categorized as their necessity of NADH, and/or NADPH [15]. These reducing agents act as an electron donor and take part in the cleavage of azo bond intracellularly/extracellularly (Fig. 1a and b) [16]. The azoreductase are differentiated into four clades I to IV and other types, majority from I to III clades are flavoproteins, and from IV are flavin free. The clades I and II are NADPH and NADH dependent, respectively while other positively used both coenzymes [17].

The different reports have showed the important role of flavin reductase in decolorization of various dyes. Flavin reductase from *Citrobacter freundii* A1 and *Sphingomonas* sp. BN6 have been reported in biotransformation of azo dye [18-20]. Various microbial species have unspecific enzymes, which work similar to azoreductase (AZR) and under anaerobic/ microaerobic condition can transfer electrons via flavin to azo dyes [18-21].

Biotransformation of various complex azo dyes by NADH-dependent azoreductase has been shown from *Alishewanella* sp. strain KMK6, *Bacillus subtilis*, *Enterococcus* sp. L2 [22-25]. The FMN-dependent azoreductase from *E. coli and*

Enterococcus faecalis has been involved in the aerobic decolorization of both sulfonated and non-sulfonated azo dyes [18,26]. Ooi et al. reported aerobic azoreductase from *Bacillus* sp. is a flavoprotein that is dependent on NADH for activity was having a broad pH and temperature stability [7]. Similarly, azoreductase of *Clsotridium perfringens* was noted for reduction of azo dye at strictly anaerobic condition [26]. On the other hand, various reports are available on the decolorization of dye in presence of NADH (low molecular weight redox mediator), where the electron shuttles among azo dyes and NADH (Fig. 1b) [16]. This broad specificity of azoreductase plays a significant role in elimination of the azo dyes containing effluents.

a



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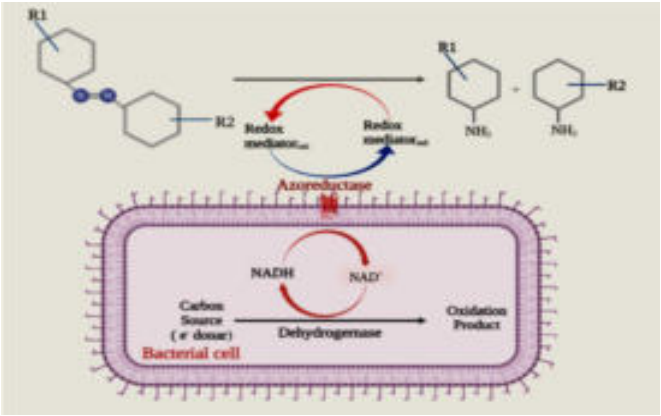

Figure-1. Mechanism of action of azoreductase in azo dyes degradation (a) general mechanism, (b) mediator dependent mechanism of azoreductase [16].

1.3.1.2. Chromate reductase

The textile effluent contains toxic heavy metals like Cr, As, Cd, Hg, etc. that were able to cause higher toxicity in presence of dyes, an important environmental concern. In bacteria, chromate Cr(VI) was reduced to Cr(III) with chromate

reductase either in the presence of some heme-protein and NADH/NAD(P)H (Fig. 2) [12,27]. Enzymatic detoxification of Cr(VI) has been reported by chromate reductase isolated from *Escherichia coli* ATCC 33456, *Lysinibacillus sp. Pseudomonas putida,* and *Lysinibacillus sphaericus* G**,** [12,28].

The literature study showed that simultaneous reduction and decolorization of chromate mediated by various enzyme system. Reduction of chromate has been described in presence of aerobic, anoxic and/or anaerobic environment. The reduction was associated with the enzyme chromate reductase and NAD(P)/NAD(P)H as electron donor in cytosol, under aerobic environment. However, in some bacteria chromate reductase activity was concomitant with plasma membrane, wherein formate and NADH act as electron donors in *Shewanella putrefaciens* MR-1. The *Shewanella oneidensis* MR-1 chromate reductase gene (SO3585) showed structural and functional similarities (28 %) with azoreductase and chromate reductase from *Bacillus subtilis* and *Pseudomonas putida* ChrR, respectively [29]. The role of nitroreductase in reduction of 2,4,6-trinitrotoluene and nitrofurazone and chromate reductase in *E. coli* DH5 and *Vibrio harveyi* KCTC 2720 have been explained earlier [30]. *Enterobacter cloacae*, a facultative anaerobe, was able to use chromate or dichromate as terminal electron acceptor in the reduction of Cr(VI) by membrane bound reduced cytochrome or dehydrogenase [27]. Chromate reductase in *Pseudomonas putida* showed quinone reductase activity, the quinone reductase from the *Rhodobacter sphaeroides* functionally showed AZR activity [24]. The noticeable induction of azoreductase and chromate reductase activities in presence of chromate has been reported, suggesting that the chromate reductase might take part in decolorization of azo dyes [12]. In contrast, Ng et al. reported the decrease in Cr(VI) reduction in presence of Acid orange 7 dye, where the reaction mediated *by Brevibacterium casei* enzyme and Cr(III) generated might have formed complex with biotransformed dye product [31].

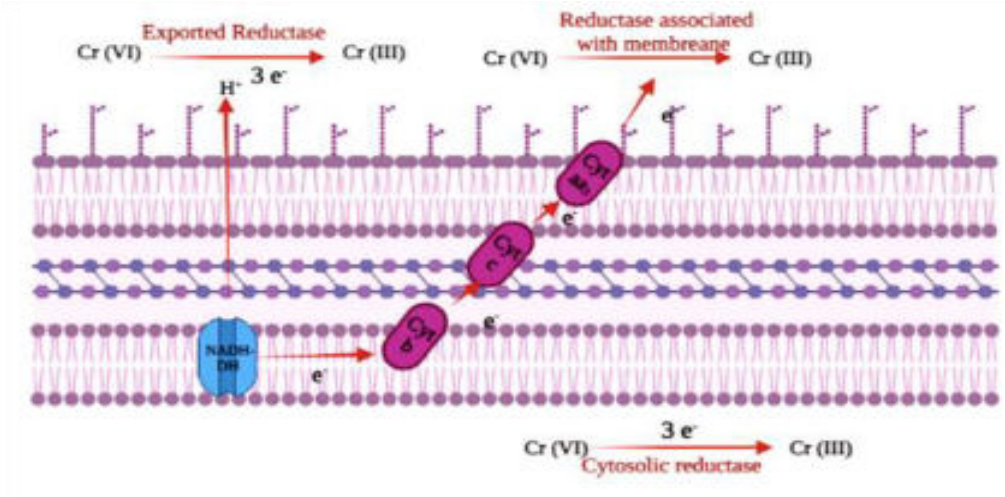

Figure-2. Pathways of Cr(VI) reduction by cytosolic reductase and membrane cytochrome/hydrogenases present in bacteria.

1.3.1.3. Cytochrome P450

Cytochrome P450 (CYPs) system has an imperative role in biotransformation of different aliphatic and aromatic contaminants. The CYPs, heme-thiolate protein belongs to a super family that perform various challenging oxidative reactions, such as N-, O-, and S-dealkylations, deaminations, peroxidations, dehalogenations, hydroxylations, and N-oxide reductions [32, 33]. The CYPs were associated with redox mediator such as transfer of electrons from NADH with flavins/Fe-S cultures to the CYPs heme center to attain high catalytic activity. Depending on variable electron transport systems, the CYPs can be divided into one- membrane bound or soluble system made with fusion of cytochrome P450 reductase [CPR] and CYP), two- CPR and CYP membrane bound, or three- protein system in which both CYP and reductase may be membrane bound or soluble [34] (Fig 3).

Due to its exceptionally diverse reaction mechanism, cytochrome P450 has an imperative role in the treatment of diverse industrial waste. Many reports suggested the involvement of cytochrome P450 in transformation of dyes and its metabolites. The studies from *B. lentus* BI377 [35], aerobic bacterial granules [11], and expression of CYP450 during decolorization of Congo red by *Lenzites gibbosa* [34] supported the presence of cytochrome P450. These findings conclude that dye and the intermediates generated during biotransformation of dye have been subsequently hydroxylated by CYP450 system (Fig. 4) and which further enters in TCA cycle [35].

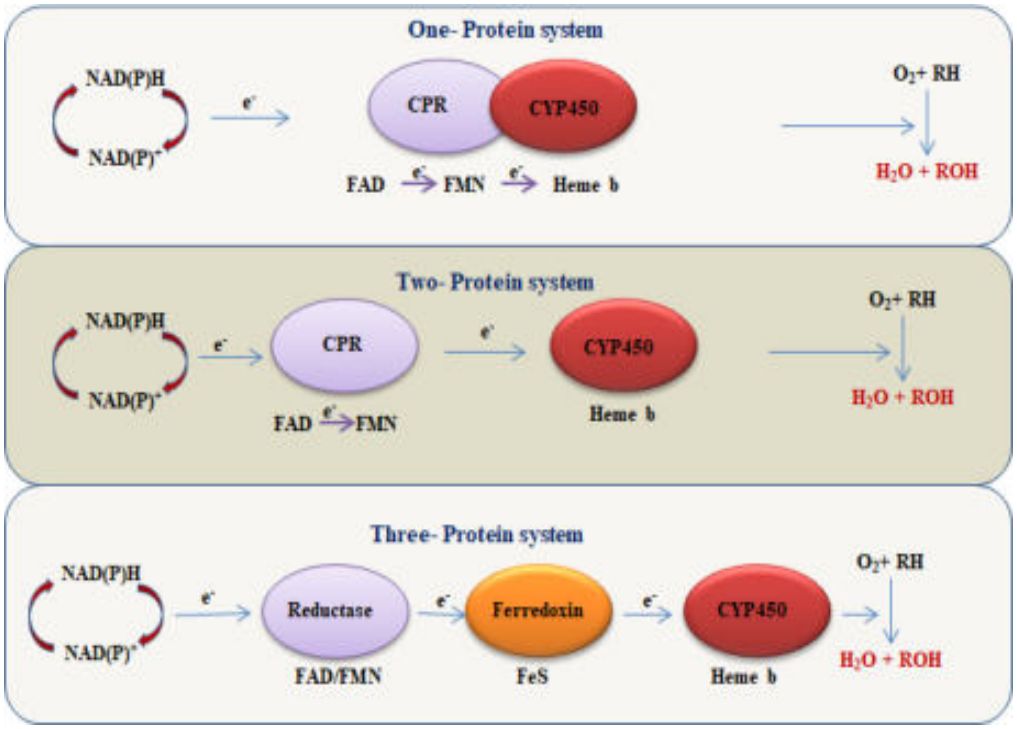

Figure-3. Cytochrome 450 Redox systems for electron transfer, One-protein system, Two-protein systems, and Three-protein systems [35].

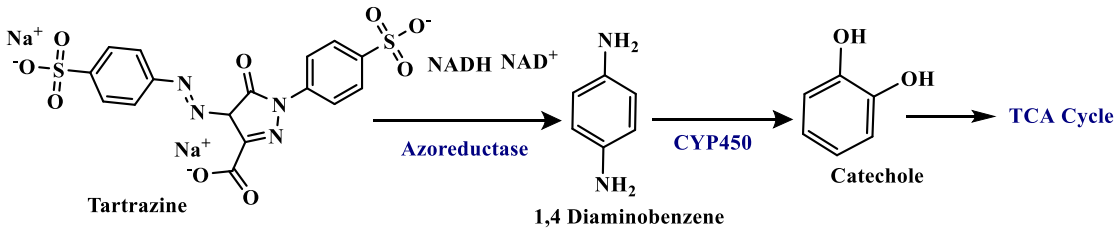

Figure-4. The proposed pathway for decolorization of dye by azoreductase and CYP450 for biotransformation of dye and its intermediate [35].

1.3.2. Laccases

Laccases (EC 1.10.3.2) are multi-copper oxidoreductase that catalyzes the oxidation of a wide range of reactions. Laccases are commonly found in fungi, plants, insects and bacteria [36, 37]. Each monomer of laccase has four copper atoms, these sites categorized into three types T1 to T3. The T1 is blue copper center where oxidation

occurs, however T2 and T3 are coupled where the O_2 reduction take place [37]. Broad substrate specificity of laccases is a distinctive feature of laccase [16]. Laccases are found to have potential in wastewater treatment generated through textile industry to decolorization and bleaching processes, where the wastewater contains lignin- or phenolic compounds and heavy metals [38]. The dyes were successively oxidized through laccase with loss of an electron and formation of nontoxic metabolites and/or water [37]. The comparative study of laccase from fungal and bacteria observed that *T. versicolor* was having 20 times more laccase activity than *Streptomyces* sp. indicating that laccase is predominantly present in fungus [39]. Wesenberg reported the involvement of laccases from *Clitocybula dusenii* in the treatment of dye effluent [40]. Laccases from *Ganoderma lucidum* was reported to have higher thermostability and decolorization activity at 60 °C and pH 4.0 [41]. Laccase from *Stenotrophomonas maltophilia* AAP56 was reported for their high redox potential (638 mV) than other bacterial lacasses [42]. These finding indicate versatile nature of laccases. Sotelo et al applied a novel approach, prepared the magnetic bio-filters immobilized with laccase on nanoparticles which improved the retention of nanoparticles in filter and stimulate the wastewater treatment by transferring mass between dye and catalyst [43].

1.3.3. Peroxidases

Peroxidases are mainly involved in catalytic oxidation of lignin and phenolics by using hydrogen peroxide. Peroxidase can be divided into heme and non-heme proteins that are widely present in the living system. Depending on their origin, peroxidases are divided on the basis of their specificity and structure as viz., animal/non-animal peroxidase, haloperoxidase, catalase, and dye-decolorizing peroxidase (DyP), di-heme cytochrome c peroxidase [44]. The DyP (E.C. 1.11.1.19) is a class II peroxidase having unique ability to oxidize to recalcitrant anthraquinone dye. The broad functional diversity of DyP was found in bacteria as DyP A, B and C, whereas in fungi, it is observed as DyP D peroxidase. Further, biodegradation of industrial dyes has been reported by DyP E along with lignin de-polymerization that occurred through oxidation of manganese/phenolics and non-phenolics compounds. The DyP has been distinctive from other peroxidases as it having a ferrodoxin like fold [45, 46]. The BaDyP peroxidase from *B. amyloliquefaciens* was observed to be effective in the decolorization and degradation of dye and phenolic ethers [30]. Lignin peroxidase (LiP) and manganese peroxidase (MnP) are multifaceted peroxidases reported due to their high degradability of toxic and waste compounds in nature. The LiPs oxidize halogenated phenolic and aromatic substances [47]. While MnP oxidized Mn(II) to Mn(III) in multistep reactions, which act as a mediator for the oxidation of different phenolics. Decolorization of Disperse Yellow 3 by *P. chrysosporium* with LiP and MnPs was well documented [48]. The combined action of oxidoreductases; azoreductase, cytochrome P450, peroxidases, and laccases with

respect to the pollutant may be advantageous for the bioremediation of dye waste under diverse environmental conditions

1.3.4. Tyrosinase

Tyrosinase (EC 1.14.18.1) is abundantly found in all domains of life. It is a copper-containing tetrameric monooxygenase having oxygen and aromatic sites and catalyzes the *o*-hydroxylation of monophenols/aromatic compounds to the corresponding catechols and subsequently to *o*-quinones [49]. Different bacteria like *Aeromonas* sp., *Pseudomonas* sp., *Sinorhizobium* sp., *Bacillus* sp., *Pseudomonas* sp., *Ralstonia* sp., *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 and fungi like *Agaricus bisporus*, *Neurospora* sp., *Lentinula* sp., *Aspergillus* sp., are reported for the presence of tyrosinase [50]. The biotransformation of dyes like malachite green, Remazol yellow RR, Methyl red were reported by tyrosinase and its metabolites were extracted and identified [50,51]. It was observed that tyrosinase along with azoreductase, and laccase plays an important role in dye degradation.

2. FUTURE PERSPECTIVES AND CHALLENGES WITH APPLICATION OF ENZYMES

The large numbers of reports are available on enzymatic bioremediation of textile dyes and its effluents. The textile dyes are stable xenobiotics that were released into the wastewater which contaminate the ecosystem. The azo and anthraquinone dyes are more toxic and were predominantly present in wastewater. The enzyme-based remediation techniques are efficient and ecological, but their lesser use in industries was due to various factors like stability, recovery, extraordinary cost and difficulties in recycling processes. The efficiency of enzymes has been improved by using immobilized enzymes with nanoparticles having a magnetic property which increases bioremediation of wastewater. Further the efficiency of enzyme increases by immobilizing it in efficient and suitable material which is having high stability and can be used in bioreactor to treat textile wastewater. Extensive exploration has been reported on the enzymatic activities, considering the remarkable accomplishments in metabolomics, further study of enzyme strategies for degradation of dye are possible. Successive actions of enzymes in the metabolic pathways are now properly explained, which will further supplement the progression of dye degradation effectively.

3. CONCLUSION

There is a big challenge to develop an effective and viable process for treatment of textile wastewater having various recalcitrant and toxic compounds. The biological methods are more sustainable, acceptable and cost effective than physico-chemical treatment methods used. The current literature showed the significant role of various enzymes in biotransformation of dyes such as oxidoreductases: azoreductase,

cytochrome P450, DCIP reductase, laccase, peroxidase, and DyPs. The real combination of oxido-reductive and other enzyme system may significantly reduce and completely remove textile dye xenobiotics. These literature studies revealed that enzymes may possibly substitute for sustainable elimination of textile dyes from effluent discharge and polluted sites.

ACKNOWLEDGMENT

The author Ashvini U. Chaudhari would like to thank University Grants Commission (UGC), New Delhi, India for Dr. D. S. Kothari postdoctoral fellowship [F.4-2/2006 (BSR)/ BL/19-20/0128] and Vishwas A. Bapat acknowledges support from the National Academy of Sciences, India for the honorary scientist fellowship.

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Antioxidant potential of *Psidium guajava* and its comparative study by using different solvents

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ABSTRACT
In nature, fruits considered as rich source of antioxidants, phenolics and flavanoids. Determination of antioxidants content can increase its market value, as the fruit become raw source of many food industries. Current study deals with the antioxidant study of Psidium guajava. Two variety of Psidium guajava are reported to study antioxidant activity as Psidium guajava (White guava) and Psidium guajava L. (Red guava). Their part was separated as peel, pulp and seed of both white and red guava respectively. Each part was extracted by using different solvents like ethanol and methanol. Antioxidants content of these samples was determined by different methods as follow; total antioxidant capacity, nitric oxide radical scavenging activity and FRAP assay (Ferric reducing antioxidant power). Wet white peel (0.93) and dry white peel (1.2) showed highest total antioxidant capacity in methanolic and ethanolic extract resp. Nitric oxide radical scavenging activity of ethanolic extract of wet red seed was highest among ethanol and methanol. It was 88.23 % scavenging activity. Wet red pulp in methanol extract had highest reading at 700 nm ie.2.7. Total phenolics were found highest in dry white peel and flavanoids content was higher in wet red pulp. This study may be useful for further research aspects of Psidium guajava and development of healthy and nutritious new food products.

KEYWORDS
Psidium guajava, *Total antioxidant capacity*, *Phenolics, Flavanoids, FRAP assay.*

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1. INTRODUCTION

Nowadays due to the increased occurrence of chronic degenerative diseases, awareness among people of their food consumption also increased. This will give people relief from the occurrence of the diseases which affects their quality of life and economic status. Some nutritional factors are drastically considered to be critical for human health. Among them, free radicals have been of concern chronic degenerative disease [1]. Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia, inflammation, metabolic disorders, heart disease, cancer, malaria, HIV/AIDS [2, 3]. Also causes injury of many tissues, a central nervous system injury, cell injury and death [4]. Due to environmental pollutants as, radiation, chemicals, toxins, deep fires, spicy foods as well as physical stress, free radicals cause depletion of the immune system. Cigarettes smoke, UV-rays and the change in gene expression induces abnormal proteins. The oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems [5]. The antioxidant activity of phenolics is mainly due to their redox properties,

which allow them to act as reducing agents, hydrogen donars, singlet oxygen quenchers and metal chelators [6].

Current research into free radicals has confirmed that food rich in antioxidants play an essential role in prevention of cardiovascular diseases and cancers [7]. A number of synthetic oxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) have been extensively added to foodstuffs, although their use has begun to be questioned because of their toxicity, so there is considerable interest in preventive medicine and in the food industry for the development of natural antioxidants obtained from botanical sources, especially herbal plants.

The food and nutrition board has defined a dietary antioxidant as a substance in commonly consumed foods that significantly decrease the adverse effects of chemically reactive species on normal physiological functions in human (food and nutrition board, 2000). Many researchers around the world have been studying antioxidant activity in various foods, especially in grains, vegetables and fruits [8].

Fruits e.g. Blueberry, grapes, cranberry, apple, plum, mango, raspberry are rich in antioxidants and can be consumed on various varieties [9, 10]. But the consumption of such fruits is limited due higher cost and seasonal availability. Thus, the common fruits such as guava, papaya, and banana were therefore investigated for total antioxidant activities and total phenolic compounds [11]. Guava (*Psidium guajava and Psidium guajava L*) is widely cultivated and its fruit is most popular in India. Guava tea, the infusion of dried guava fruits and leaves, has recently become popular as a drink in Taiwan. Guava fruit is considered a highly nutritious fruit because it contains a high level of ascorbic acid (50.300 mg/100 g fresh weight), which is three to six times higher than oranges [12]. Indonesian guava is an excellent source of provitamin A caratenoids: phenolic compounds such as myricetin and apigenin, ellagic acid, and anthocyanins are also at high levels in guava fruits [13, 14]. The appearance and morphology of guava tree and fruit as shown in fig. 1 and 2A; 2B respectively. The classification of guava is as follows;

Classification:

Kingdom: plantae-plants

Subkingdom: Tracheobionta-vascular plants

Superdivison: Spermatophyta-seed plants

Division: Magnoliophyta-flowering plants

Class: Magnoliopsida-dicotyledons

Subclass: Rosidae

Order: Myrtales

Family: Myrtaceae-Myrtle family

Genus: *Psidium* L-guava

Species: *Psidium guajava* L-guava

Therefore, producing guava especially bread for higher levels of antioxidant compounds is a realistic approach to increase dietary antioxidants intake. Evolution in any plant breeding program, however, has to deal with numerous plants, particularly at the early selection stage.

Therefore, the assay for screening, inexpensive, rapidly performed, and provide a high degree of precision.

Current study focuses on extraction of different parts of white and red guava variety by using different solvents. Estimation of total antioxidant capacity, phenolics, flavanoids, nitric oxide radical scavenging activity and FRAP assay (Ferric reducing antioxidant power) and its comparative analysis.

Figure-1. Image of *Psidium guajava* tree

Figure-2A. Vertical section of white guava.

Figure-2B. Vertical section of red guava.

2. EXPERIMENTAL SECTION

2.1. Collection of Plant material

The plant materials (red and white guava) were collected from market. Samples were dried for 8 days and these dried samples as well as wet samples were used for extraction

2.2. Preparation of extracts

The dried and wet samples were extracted using two different solvents as,

1) Extraction by ethanol.

2) Extraction by methanol.

The various sample from red and white guava were taken for extraction as,

a) Wet white seed.

b) Wet white peel.

c) Wet white pulp.

d) Dry white seed.

e) Dry white peel.

f) Wet red peel.

g) Wet red pulp.

h) Wet red seed.

2.2.1. Ethanol extraction-

The 4gms of dried & wet samples were weighed and crushed into mortal pestle using 50% aqueous ethanol (8 ml) followed by centrifugation at 10,000 rpm for 15 min at 5°C. The mixture was homogenized was collected, stored at 4°C for further use.

2.2.2. Methanol extraction-

The given 8 samples of guava were weighed 3 gm of each and crushed into mortal pestle. After crushing, 25 ml of methanol is added to it. The mixture was homogenized by using homogenizer. These mixes were kept for centrifugation at 15000 rpm for 20 min at 5°C. Then the supernatant was collected and was stored at 4°C for further use.

2.3. Antioxidant Assay

2.3.1. Total phenolic content-

The total phenolic content of different extracts was measured using colorimetric Folin-Ciocalteu method. The reaction mixture consisted 5 ml of diluted sample to which 3 ml of distilled water and 0.5 ml Folin-Ciocalteu reagent was added [15]. After 3 mins, add 2 ml of 20% NazCO, solution and place the tubes in boiling water bath for one mins, cooled and the absorbance was measured at 760 nm. Standard graph was plotted by taking different concentration of pyrocatechol.

2.3.2. Total antioxidant capacity-

0.1 ml of extract was combined in eppendrof tube with T ml of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in thermal block at 95 °C for 90 mins. After cooling to R.T; the absorbance of aqueous solution of each was measured at 695 nm against blank [16].

2.3.3. Total flavonoids content-

The flavonoids content of different extracts was measured using a modified colorimetric method. 0.5 ml of sample was mixed with 0.5 ml of 2% AICI3 for 10 mins and the absorbance was measured at 415 nm. The measurement was compared to a standard graph for curcumin [17].

2.3.4. Nitric oxide radical scavenging activity-

Sodium nitroprusside (5mM, l ml) phosphate buffer saline (PSB) (0.1 M, 7.4 pH) was mixed with 3 ml of different concentrations of the extract and incubated at 25° C for 15 min. 0.5 ml of Griess reagent (1% sulphanilamide, 2% H3PO, 0.1% napthylethylenediamine dihydrochloride) was added. Measure the absorbance at 546 nm [18]. The percentage scavenging calculated by formula as follows:

% Scavenging = A (conc.)-A (test) /A (conc.) * 100

2.3.5. Determination of Ferric reducing antioxidant power (FRAP)-

Various concentration of extracts in 1 ml of water were mixed with Phosphate buffer (2.5ml, 0.2 M pH 6.6) and 1% Potassium ferricyanide (2.5ml). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5,10%) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and freshly prepared FeCl3 solution (0.5ml,0.1%). The absorbance was measured at 593 nm [19].

3. RESULTS AND DISCUSSION:

3.1. Total phenolic Content (TPC) -

The phenolic compounds present in the extract were detected using colorimetric Folin-Ciocalteau method. Phenolic acids are plants metabolites widely spread throughout the plants kingdom. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases (coronary heart diseases, stroke and cancers). Phenolic compounds are essential for growth and reproduction of plants and are produced as a response for defending injured plants against pathogens. In the assay carried out, pyrocatechol acid was used as standard.

Total phenolic compounds were reported as pyrocatechol equivalent. From the std. graphs of pyrocatechol total phenolic contents, TPC of ethanolic extracts range from 0.03 mg/ml to 0.067 mg/ml and that of methanolic extracts range from 0.06 mg/ml to 0.081 mg/ml. From results it can be revealed that sample wet white peel and wet white seed (0.081) showed highest TPC as shown in **Table-1** and **Table-2**.

Table- 1. Total phenolic content of ethanolic extract of *Psidium guajava.*

Sr. No	Ethanol extract	Phenolic content in Mg/ml
1	Wet white seed	0.057

2	Wet white peel	0.067
3	Wet white pulp	0.058
4	Dry white seed	0.056
5	Dry white peel	0.062
6	Wet red peel	0.031
7	Wet red pulp	0.052
8	Wet red seed	0.033

Table-2. Total phenolic content of methanolic extract of *Psidium guajava.*

Sr No	Methanol Extract	Phenolic content in mg/ml
1	Wet white seed	0.081
2	Wet white peel	0.068
3	Wet white pulp	0.054
4	Dry white seed	0.045
5	Dry white peel	0.033
6	Wet red peel	0.006
7	Wet red pulp	0.042
8	Wet red seed	0.039

3.2. Total antioxidant capacity-

Total antioxidant capacity of different samples of guava using two different extract is shown in **Figure-1**. In this assay ethanol extract was found to have higher activity than methanol extract. Wet white peel showed highest total antioxidant capacity. The extract demonstrated electron donating capacity and thus they may act as radical chain terminators, transformating reactive free radical spp. into stable non-reactive product.

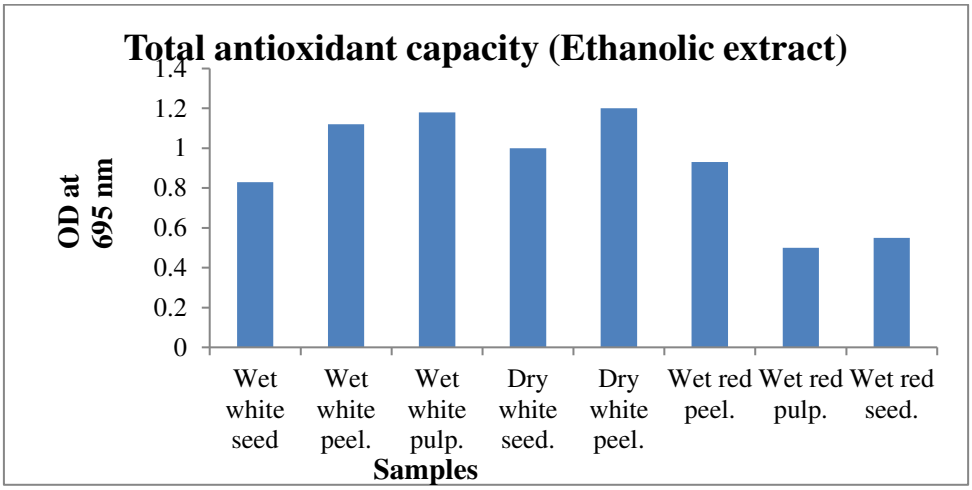

Figure-3. Total antioxidant capacity of ethanolic extract of *Psidium guajava.*

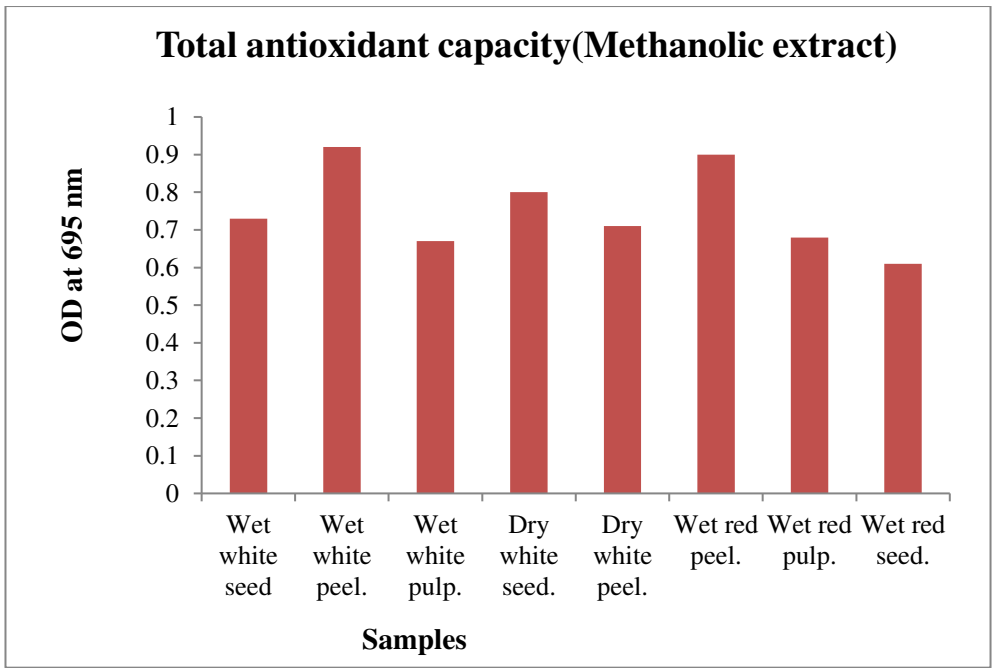

Figure-4. Total antioxidant capacity of methanolic extract of *Psidium guajava.*

3.3. Total Flavanoids-

It has been reported that green leafy vegetables, soft fruits and medicinal plants exhibited higher levels of flavanoids. The poly phenolic compounds such as flavanoids have been shown to possess significant antioxidant activities. The total flavanoid contents of different samples were measured using modified colorimetric method. TFC was measured as µg/curcumin equivalent per gram of samples. TFC content of methanolic extract range from 0.06mg/ml to 0.081 mg/ml and ethanolic extract range from 0.03 mg/ml to 0.06mg/ml.

Ethanol extract had higher flavanoid contents than methanol extracts wet white peel and wet white pulp showed significantly higher flavanoids content equivalent to curcumin.

3.4. Nitric oxide radical scavenging activity-

The extract successfully reduced generation of nitric oxide from sodium nitroprusside. In vitro inhibition of nitric oxide radical is the measure of antioxidant activity of plant. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which results with oxygen to produce nitrite ions that can be measured by using Griess reagent. Both ethanol and methanol extract decreased the amount of nitrite generated from decomposition of sodium nitroprusside invitro which may be due to the presence of antioxidant principles in the extract. There were no significant difference ($p>0.05$) between ethanol and methanol extracts. The percentage scavenging activity of ethanol and methanol extract is shown in the table below. The % scavenging activity was relatively high for ethanolic extracts then for methanolic extracts wet red seed **(Table-3)** and wet red peel **(Table-4)** showed highest nitric oxide radical scavenging activity.

Table-3. Nitric oxide radical scavenging activity of ethanol extract of *Psidium guajava.*

Samples	**% Scavenging Activity**
Wet White seed	19.41
Wet White Peel	65.41
Wet White Pulp	62.58
Dry White Seed	17.41
Dry White peel	68.82
Wet red Peel	86.82
Wet red pulp	83.76
Wet red seed	88.23

Table-4. Nitric oxide radical scavenging activity of methanol extract of *Psidium guajava.*

Samples	**% Scavenging Activity**
Wet White seed	42.21
Wet White Peel	42.63
Wet White Pulp	20.52
Dry White Seed	16.21
Dry White peel	18.10
Wet red Peel	63.89
Wet red pulp	63.26

Wet red seed	65.57

3.5. Determination of Ferric reducing antioxidant power (FRAP):

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of measuring the formation of Perl's Prussian blue at 593 nm. Increasing absorbance at 593 nm indicates an increase in reductive ability .Below graph shows reducing powers activity of methanolic and ethanolic extract respectively. There is no significant difference among the ethanol and methanol extract in reducing power. Dry white seed (2.4) and wet red pulp (2.7) shows highest reducing activity as shown in **Figure-5** and **Figure-6**.

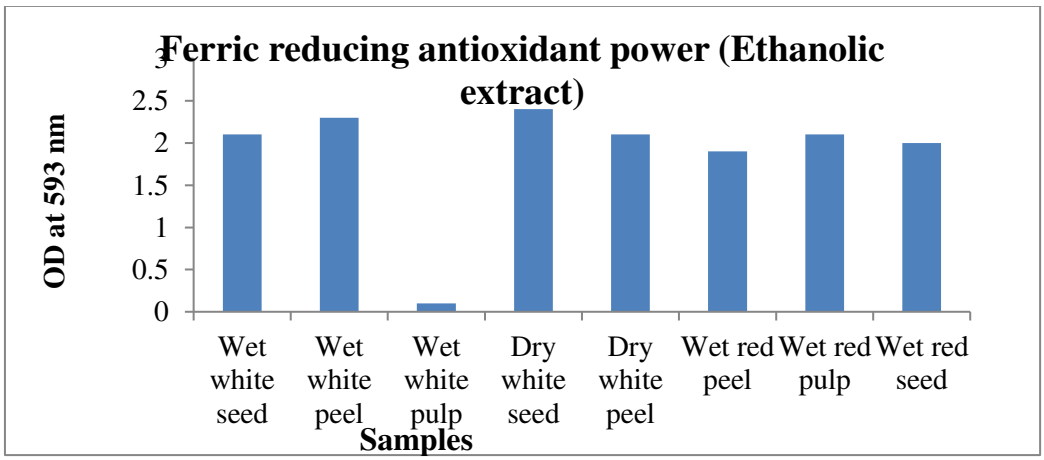

Figure-5. Ferric reducing antioxidant power ethanolic extract of *Psidium guajava.*

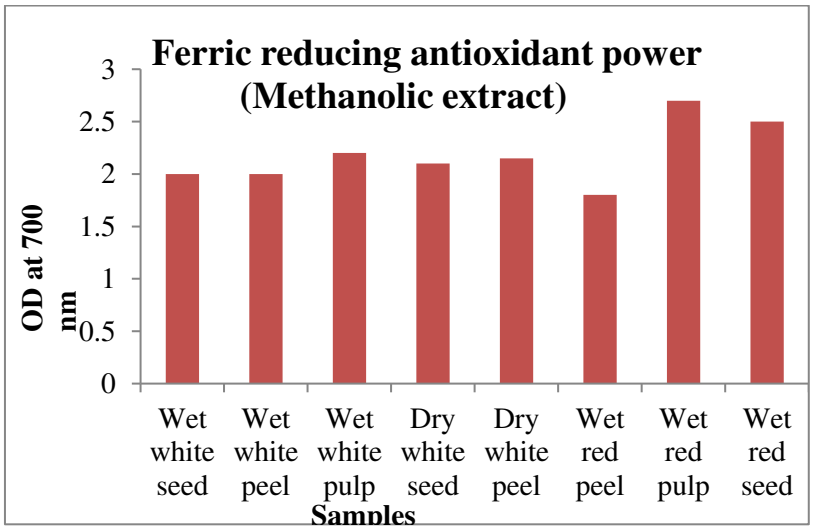

Figure-6. Ferric reducing antioxidant power methanolic extract of *Psidium guajava.*

4. CONCLUSION

Antioxidants are considered as scavengers of free radicals. Free radicals exist as independent molecular ssp. generated by metabolism and environmental effects. Synthetic antioxidant such as BHA, have restricted use in foods as these synthetic antioxidants are suspected to be

carcinogenic. Therefore importance in exploitation of natural antioxidants especially of plant origin has greatly increased in recent years. Aim of our project was to evaluate antioxidant capacity of ethanolic and methanolic extracts of white and red guava. The results revealed that ethanolic extract showed highest antioxidant capacity than methanol. In most cases wet white fruit extracts showed highest antioxidant activity than dry extracts. Thus people should include natural sources of antioxidant like fruits (guava) in their diet. Hence antioxidants are important life supporting compounds.

ACKNOWLEDGMENT

The authors are thankful to Department of Biochemistry, Shivaji University, Kolhapur for awarding Diamond Jubilee Post Doctoral Fellowship and providing all research facilities to carry out this work.

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Novel Biogenic Gold Nanomaterial Synthesised from Seed Extract of *M. Imbricata* for Anti-Bacterial and Anti-Antioxidant Potential

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ABSTRACT

The current report focused on the biogenic synthesis of novel gold nanoparticles (AuNPs) from Mucuna imbricata seed extract. The synthesis conditions were optimized for higher yield of AuNPs with uniform size distribution. The Mucuna species has attracted a lot of attention recently because of its potential medicinal, agronomic, and nutritional benefits. Produced nanoparticles demonstrated for antioxidant potential having free radical species scavenging ability and oxidative stress lowering capacity which is a major parameter in the management of degenerative diseases. The M. imbricata water was used for the reduction of gold salt at different dilutions at ambient synthesis conditions. The prepared nanoparticles are thoroughly evaluated for morphological, structural, functional and optical properties using various characterization techniques like UV visible spectroscopy, X-ray diffractometer (XRD), Fourier Transform Infra-Red (FT-IR) Spectroscopy, and Transmission Electron Microscopy (TEM). The results of FTIR, XRD and TEM analysis showed the synthesis of spherical, mono-dispersed, crystalline nanoparticles having maximum absorption at 550 nm under UV visible spectroscopy. The results of DPPH and ABTS assays confIrmed the antioxidant potential of biogenic AuNPs along with better antibacterial potential against Bacillus cereus and E. coli. Herein synthesized biogenicAuNPs are having strong antimicrobial and antioxidant potentials. Since it shows noteworty activity in free radical scavenging which can be further studied for the controlling of Parkinson's disease.

KEYWORDS

Antioxidant activity, FTIR, Gold-nanoparticles, M. imbricata, TEM.

1. INTRODUCTION

Nanotechnology is an interdisciplinary field that is related to atomic and molecule levels of matter regulation and current scopes of nanotechnology is focused on food technology, medicine, agriculture, and the health are some of the major domains where nanotechnology has been mainly used [1]. Physical and chemical synthesis processes are not always the best options due to the numerous drawbacks such as excessive energy consumption, pollution, and health hazards [2]. Green chemical synthesis principles serve as viable options for researchers looking to develop innovative green synthesis methods. [3,4]. The plant is a rich source of bioactive agents, polymers and other biological entities which will help in the production of NPs with a precise shape, size and content the presence of a diverse range of phytochemicals in their extracts could operate as a natural stabilizer and/or reducer for nanoparticles. However various physical applications were also studied from nanomaterial produced using cost effective methods. [5,6]. Mucuna is a well-known medicinal plant for Parkinson's disease management for its potential effect on bioactive compounds and nutritional content. The previous study on nanoparticles preparation by reduction of silver ions by M. pruriens seed extract [7]. However, preparation and characterization of gold nanomaterial using M. monosperma were studied by Vora et. al. in 2010 showed good stability [8]. Ali et. al. (2021) synthesized copper nanoparticles from M. pruriens seed extract exhibiting strong antioxidant actions in free radical scavenging employed in a clinical therapeutic application, antidiabetic activity [9].

Pawar et al. (2021) explored a method for nanomaterials using *Murraya koenigii* leaf extract by reduction using silver nitrate at ambient temperature, which is stable over several months according to spectrophotometric examination. [10]. Bos taurus urine-assisted nanoparticles prepared from palladium and gold were previously prepared and elaborated for their biological activity [11,12]. Previous literature on *Mucuna imbricata* explained the potentials of phytochemical activities and biological activity of seed extracts and callus produced from stem explant [13-15]. Whereas synthesis of silver-decorated magnetic particles, *Azadirachta indica* leaves extract selenium nanoparticles, and copper ferrite magnetic nanoparticles were studied previously by our colleague Otari in their literature [16-18].

In this report, we present an economically feasible method for the preparation of gold nanoparticles from *Mucuna imbricata* seed extract. Different analytical methods, including transmission electron microscopy (TEM), X-ray diffraction

(XRD), Fourier transform spectroscopy (FTIR) and UV-Vis spectroscopy analysis was employed to examine the crystal structure, optical properties and morphology of the generated nanomaterials. Properties like antibacterial activity against various pathogenic bacterium and antioxidant potential were observed demonstrated in the prepared nanoparticles which will be useful in food and medicine.

2. EXPERIMENTAL SECTION

2.1. Reagents and standard chemicals

All of the compounds were of analytical grade and had a high degree of purity for this study. From Sigma, 2, 4-, 6-tripyridyl-s-triazine (TPTZ), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and quercetin hydrate were purchased (St Louis MO, USA). Other chemicals, including gold chloride, ferric chloride, and gallic acid, were bought from Himedia in Mumbai, India. Solvents for extraction were of HPLC grade purchased from S D Fine-Chem Ltd, whereas, standards and solvents used in a study like methanol, ascorbic acid, gallic acid, and HPLC pure water having HPLC grade as per requirement were purchased from Spectrochem Pvt. Ltd. India.

3. 1 Synthesis of gold nanoparticle nanomaterial from *M. imbricata* extract.

Synthesis of gold nanomaterial from *M. imbricata* seed extract was done by using the method as per the description [19,20]. Biogenic production of gold nanomaterial was performed by using a 100 µL of 1 mM chloroauric acid (Gold (III) chloride) 10^{-3} M of concentration which was prepared by using deionized distilled water. Different concentrations of stock solution of chloroauric acid solution and *M. imbricata* seed extract (1:99, 2:98, 3:97, 4:96, and 5:95) were used. The reduction reaction of gold ions using *M. imbricata* extract to gold nanomaterial was done within 5-10 min of stirring. The change in the color due to the reduction of metal ions was measured continuously by measuring UV–visible spectrometer spectra in the wavelength range 200 – 800 nm. The color change is the main sign of the development of gold nanomaterial which was conformed using UV spectrophotometric technique, where, other analytical techniques were used for characterization and checking surface morphology. Optimization of nanomaterial synthesis was done by using variations in stock concentration of gold chloride and amount of *M. imbricata* seed extract with change in shaking period.

3.1. Characterization of gold nanoparticles prepared using *M. imbricata*

The prepared *M. imbricata* seed extract nanoparticles were characterized for their UV-Vis analysis which was performed by using UV-Vis double beam spectrophotometer. XRD study was performed to reveal the structural nature of the nanomaterial produced. The typical X-ray generator configuration of 40 Kev and 30 mA was used to store data and a 0.1540 nm wavelength CuK X-ray beam. Its structure was determined using XRD which was carried out in a wide range of 20 - 80^{o}. The translational symmetry, in particular the size and shape of the unit cell, is explained by the peak location. The Scherer formula can be used to determine the crystal size.

FTIR was used for studying conjugation studies, newly manufactured gold nanomaterial was centrifuged at 8,000 RPM for ten min, however, the suspension produced was redisposed in the purified deionized water. Pellets produced were dehydrated and smirked with KBr pellets and examined under Thermo Nicolet Quator apparatus in the diffuse reflectance method working at a resolution of 4 cm^{-1}. TEM micrographs were taken on for surface characteristics of the nanomaterial using JEOL model 1200 EX, which measure particle size and surface morphology of nanoparticles at an accelerating power of 80 keV. The synthesized gold nanomaterial was mixed by dropping a few droplets of the gold nanomaterial mixture on Lacey carbon grids, 300 mesh.

3.2. Application of gold nanoparticles prepared from *M. imbricata*.

3.2.1. Minimal inhibitory concentration (MIC)

MICs of prepared gold nanoparticles were assessed using the broth microdilution method [21], Working solutions of various concentrations, such as 50 µg/mL, 100 µg/mL, 150 µg/mL, and 200 µg/mL, were created using the gold nanoparticles synthesised from *M. imbricata* master stock of 1 mg/mL. In this experiment, fresh inoculums of four bacterial strains *Salmonella typhimurium* (NCIM 5278), *Escherichia coli* (NCIM 2662), *Pseudomonas aeruginosa* (NCIM 2037), and two gram-positive strains *Staphlococcus aureus* (NCIM 5276) and Bacillus cereus (NCIM 2217) and three Gram-negative strains Salmonella. All bacterial strains were individually inoculated into 3 mL of nutritional broth containing 100 µL of different concentrations of *M. imbricata* gold nanoparticles, and then the mixture was shaken at 100 RPM for 20–24 hours at 37°C. Using a UV-Visible spectrophotometer to measure each tube's optical density at 625 nm, microbial growth suppression or

stimulation was detected. Sterile distalled water served as the experiment's negative control, while antibiotic streptomycin, with a concentration of 50 µg/mL, was employed as the experiment's positive control.

3.2.2. Agar well diffusion techniques

1. Antibacterial potential

Simple agar well diffusion methods were studied to check the bactericidal properties of *M. imbricata* extracts [22,23]. The National Chemical Laboratory in Pune provided the microorganisms for this investigation, including *Bacillus cereus* (NCLM2703), *Escherichia coli* (NCLM2832), *Proteus vulgaris* (NCLM2813), *Salmonella typhimurium* (NCLM2501) and *Pseudomonas aeruginosa* (NCLM5032) on nutritional agar slants, the stock cultures were maintained at a temperature of 37 °C. A loopful of microbial culture was inoculated in 3 mL of saline solution to create the new working cultures, and 0.5 mL of this active suspension was dispersed aseptically on the nutritional agar plate using a sterile cork borer. Each labelled well received 100 µL of aseptically added nanomaterial extracts, and plates were then placed in the freezer for 20 minutes to facilitate extract diffusions before being incubated at 37 °C for 48 hours. Plates were observed for the appearance of inhibitory zones around the wells with the sample. Streptomycin antibiotic of concentration 100 µg/mL was used as a reference substance.

2. Antifungal activity

The effectiveness of several nanomaterials as antifungal agents was assessed using the well diffusion method. The PDA plates were made using submerged inoculations of two fungus strains: *Fusarium solani JALPK* [24] and *Aspergillus niger* (NCIM 1360). This method is similar to that employed in the agar well-diffusion approach in antibacterial activity. These agar plates were preserved for diffusion and incubated at 37 °C after being filled with nanoparticles (100 µL) of various concentrations. The examination of the zone of inhibition and took a mm-based measurement of the well size.

3.2.3. Antioxidant activity

1. ABTS radical scavenging assay

According to the older method, the ABTS radical cation decolorization assay was frequently employed to quantify free radical scavenging activity [25]. 0.5 mL of *M. imbricata* nanoparticle sample of concentration 1 mg per ml was allowed to react with 1.5 mL ABTS reagent and absorbance was measured at 734 nm. The oxidation

of ABTS with potassium persulfate in this procedure led to the production of the ABTS radical. Methanol of purity 99.5% was considered as a blank solution (A control) whereas, ascorbic acid was considered as a positive standard. After 2 hours of incubation, the decrease in absorbance was observed. The following formula was used to get the inhibition percentage:

$$\text{ABTS radical scavenging effect (\%)} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \text{ X } 100$$

Where A control is the absorbance of ABTS radical + methanol.

A sample is the absorbance of ABTS radical + *M. imbricata* nanoparticle extract.

4. DPPH radical scavenging assay

DPPH free radical scavenging potential of *M. imbricata* nanoparticle samples was demonstrated in a modified DPPH radical scavenging assay [26] with modifications as per the convenience of the sample. 200 mL of sterile distilled water were added to 60 mL of the nanoparticle extract at a concentration of 1 mg per ml. It was filled with 3 mL of freshly made methanolic DPPH solution (0.1 mM, 5.0 mL), thoroughly mixed, and left to stand for 30 minutes at room temperature in the dark without any light. DPPH scavenging activity was deliberate using the formula:

$$\text{\% DPPH scavenging activity } = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

Where;

A control: Absorbance of the initial concentration of DPPH

A sample: Absorbance of the remaining concentration of DPPH

5. RESULT AND DISCUSSION

5.1. Biogenic synthesis of gold nanoparticle from *M. imbricata* seed extract

Biogenic production of gold nanomaterial was performed by a 100 µL mixture of one mM chloroauric acid at a concentration of 10^{-3} M made by dissolving in deionized distilled water. Diverse concentrations of stock solution of $HAuCl_4$ solution of *M. imbricata* water seed extract (1:99, 2:98, 3:97, 4:96, and 5:95) were subjected, respectively. The reduction of gold ions using *M. imbricata* extract to gold nanoparticles was done within 5-10 min of stirring. The extract solution's hue changes to ruby red, which was the main sign that gold nanoparticles had formed. The characterization of produced nanomaterial is very significant because it conforms production of synthesized nanomaterial morphology. We have employed

spectroscopic and microscopic techniques to analyse the nature and morphologies of the nanoparticles because they are too small for the human eye to see.

5.1.1. UV-Visible Spectroscopy

Following the successful production of nanoparticles, the dispersed nanomaterial solution was placed in a quartz cuvette and exposed to UV-visible radiation in order to check absorption. Maximum absorption was seen here at 550 nm, which was consistent with values for gold nanomaterial that have been reported. (**Figure-1A**). UV- Visible Spectrometry is a generally used and reusable method for the characterization of prepared nanoparticles.

5.1.2. FTIR (Fourier - transform infrared spectroscopy)

FTIR spectroscopy of M. imbricata gold nanoparticles. showed (**Figure-1B**) the spectra acquired from the prepared nanomaterial in the range of 600 – 4,000 cm−1. The FTIR of M. imbricata seed extract gold nanoparticles were observed at 3851, 3740, 3327, 2365, 2314, 1638, 1550, 1468, 1260 and 603 cm−1. The O-H stretch, which indicates the presence of phenols, flavonoids, benzophenones, and anthocyanins, is where the greatest stretching appears at 3000–3500 cm−1 [27]. The region of 1638 cm−1 indicated the presence of C = O stretching whereas the region of 1600 – 1500 cm−1 suggests a C - C in ring aromatic bond which determines the presence of aromatics structure that exists in the *M. imbricata* water extract that might be due to the phenolic and other secondary metabolites. The C-C aromatics stretch was seen in spectra with wavelengths between 1500 and 1400 cm−1.

5.1.3. 3.3.3 XRD study

The XRD pattern for M. imbricata water extracts gold nanomaterial is shown in **Figure-2A**, obtained between a wide and narrow range of 2θ value i.e. from 20 to 85°. Total the diffraction points detected can be allotted to the (111), (200), (220) and (311) diffraction peaks of structures. A significant (111) diffraction peak recommends a noticeable development of network structural assembly along (111) planes likened to (200). Moreover, the additional prominent diffraction peak (220) points to the an isotropic (network) nature of the nanoparticles. Whereas, the peak strengths in particular proved the cell's electron density. Therefore, the XRD approves the crystalline nature of gold nanomaterial. Our XRD results are similar to gold nanoparticles produced from *Pergularia daemia* by Rajendran et al (2017) and extract of *Garcinia mangostana* fruit peels by Xin Lee et al (2016).

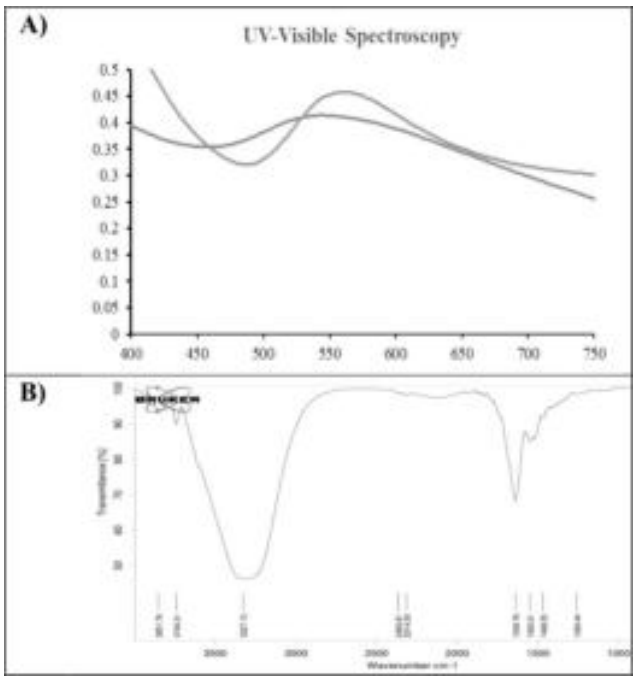

Figure-1. A) Spectrophotometric absorbance at 400 to 750 nm by UV- Visible Spectroscopy and B) FTIR spectra (600–4,000 cm”1) of *M. imbricata* seed extract gold nanoparticles.

5.1.4. 3.3.4 TEM study (Transmission electron microscope)

Results of the TEM analysis is presented in the given **Figure-2B**, discovered that there was no indication of agglomeration in elements that were round and well dispersed. An enlarged vision of a gold nanomaterial is accessible in a characteristic high resolution. It displayed the occurrence of circular mono-dispersed nanomaterial with element sizes ranging from 10 to 20 nm as revealed in **Figure-2B**. This discovery is in line with the earlier outcome attained [28,29] on the TEM description of gold nanoparticles manufactured from *Periploca aphylla* and *Annona muricata* respectively.

5.2. Determination of biogenic activities

In the current investigation, the anti-oxidant activity of the produced nano particles and antibacterial assessed using two different methods. The outcomes from the two experiments are in strong accord with one another.

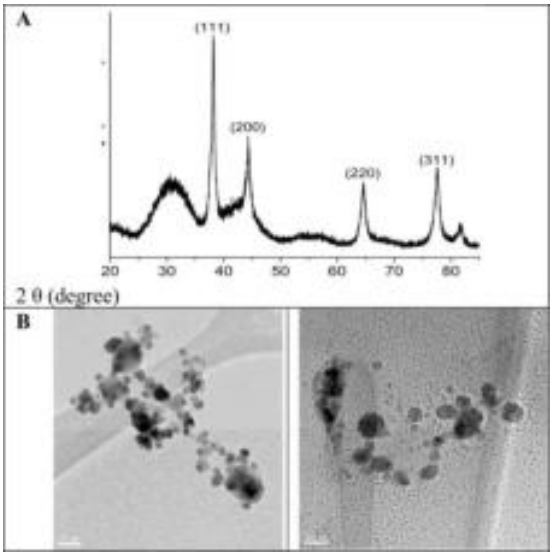

Figure-2. A) XRD (range of 2θ value) and B) TEM (20 nm and 50 nm) characterization of gold nanoparticles prepared from *M. imbricata* seed extract.

5.2.1. Determination of minimum inhibitory concentrations (MIC)

Gram-positive and gram-negative bacteria are both resistant to AuNPs, according to the antibacterial potential of solutions with various concentrations of the particles. The MIC of *M. imbricata* water extracts gold nanoparticles were studied using a spectrophotometric method [25], where turbidity was measured at spectrophotometric absorbance at 625 nm. No growth was observed under the spectrophotometer at a concentration of 200 µg/mL which is a higher concentration in the given study. This means that a 200 µg/mL concentration exhibits bactericidal action, which is crucial for creating antibacterial agents. Results show whether there is turbidity or not, which is shown in the table by + or -, accordingly. The findings showed that *Bacillus cereus* and *Escherichia coli* strains had lesser bactericidal efficacy, as evidenced by visible growth, whereas *Pseudomonas aeruginosa* and *Salmonella typhi* were sensitive to nanoparticles.

Conc µg/mL	*Escherichia coli*	*Salmonella typhi*	*Bacillus cereus*	*Pseudomonas aeruginosa*
25	+	+	+	+
50	+	+	+	+
100	+	+	+	+
150	-	-	+	+
200	-	-	-	-

Positive (+): Presence of turbidity in the medium due to microbial growth,
Negative (-): Absence of turbidity in the medium due to no growth.

Similar results of MIC were observed with Brazilian red propolis prepared gold nanoparticles for their antimicrobial and anticancer study in a prior report [30]

5.2.2. Antibacterial activity and antifungal activity

Results revealed that synthetic nanoparticles have fairly effective antibacterial properties against *Bacillus cereus* and *E. coli*. As shown in **Table-2**, the antibacterial and antifungal effects of *M. imbricata* seed extract gold nanoparticles were assessed against a collection of four bacteria and two fungi. By determining inhibitory zones were present or not, their potency was qualitatively evaluated (zone diameter in mm). **Table-2** results show that the extract of nanoparticles at the specified doses has significant antibacterial activity against the tested pathogens. Our results are in agreement with previously reported gold nanoparticles prepared from *C.lanatus* [31]. Despite the fact that *P. aeruginosa* and *S. typhi* showed good antibacterial activity, the zones of inhibition for both microorganisms were found to be 16 mm. Less antimicrobial spectrum was found in *A. niger* than *F. solani* which indicates that the *M. imbricata* seed extract gold nanomaterial has the lowermost antifungal action. effects of new antimicrobials that depend on size in M. imbricata seed water extract gold nanomaterials are earlier studied by Osonga et al(2020) [32], which are used as potential fungicides.

5.2.3. Antioxidant potential of gold nanoparticles

Commonly employed or practical ways to assess the destructive potential of free radicals include ABTS and DPPH in *M. imbricata* seed extract gold nanomaterials are shown in **Figure-3A** and **Figure-3B**. Biogenic preparation of gold nanoparticles has a different application with various methods of preparation as reported previously by Ahmed et al (2016) and Sharma et al. (2007) [33,34]. Significantly, biogenic synthesis of *M. imbricata* water extract gold nanomaterial demonstrates a good antioxidant action; therefore, it signifies capability to antioxidants with probable application in biomedical compounds preparation. Biogenic preparation of gold nanoparticles has a different application with various methods of preparation as reported previously by Ahmed et al (2016) and Sharma et al. (2007) [33,34] Significantly, biogenic synthesis of *M. imbricata* water extract gold nanomaterial demonstrates a good antioxidant action; therefore, it signifies capability to antioxidants with probable application in biomedical compounds preparation.

Table-2. Anti-bacterial and anti-fungal activity of *M. imbricata* seed extracts gold nanoparticles on various test microbes.

		Bacterial strains				Fungal stains	
Sample name	Conc µg/mL	*Escherichia coli*	*Salmonella typhi*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Aspergillusn iger*	*Fusariums olani*
M. imbricata gold nanoparticles	50	10 ± 0.51	12 ± 0.35	13 ± 0.7	11 ± 0.34	10 ± 014	10 ± 0.35
	100	13 ± 0.78	14 ± 0.9	14 ± 0.51	12 ± 0.24	10 ± 0.25	11 ± 0.84
	150	14 ± 0.74	15 ± 0.55	14 ± 0.76	14 ± 0.63	11 ± 0.47	12 ± 0.8
	200	15 ± 0.4	16 ± 0.47	15 ± 0.6	16 ± 0.47	12 ± 0.65	11 ± 0.7
Streptomycin	50	19 ± 0.47	20 ± 0.51	22 ± 0.43	22 ± 0.81	20 ± 0.74	21 ± 0.47

Biogenic preparation of gold nanoparticles has a different application with various methods of preparation as reported previously by Ahmed et al (2016) and Sharma et al. (2007) [33,34]. Significantly, biogenic synthesis of *M. imbricata* water extract gold nanomaterial demonstrates a good antioxidant action; therefore, it signifies capability to antioxidants with probable application in biomedical compounds preparation. The potentials of *M. imbricata* for their antioxidant and antimicrobial potency with the use of different solvents have been demonstrated in this study.

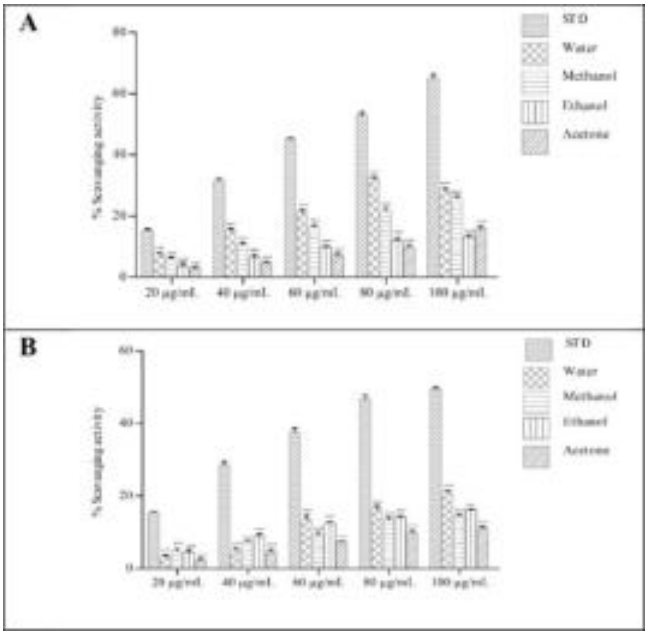

Figure-3. Antioxidants potential A) DPPH and B) ABTS potential of different concentrations of nanoparticles compared with standard ascorbic acid.

6. CONCLUSIONS

An increased interest in environmentally friendly methods has been sparked by the use of green chemistry and the growing awareness of green chemistry. Herein we have reported a new nanomaterial prepared from *M. imbricata* seed extract for the synthesis of gold nanoparticle which were rapid, energy efficient, eco-friendly and inexpensive. Analytical tools like FTIR, UV-Visible spectrophotometer, XRD and TEM helped to understand the surface chemistry of the gold nanoparticles. Study concluded that prepared nanoparticles was in very small-time span, small in size and crystalline in nature. Prepared nanomaterial exhibited enhanced antioxidant and antimicrobial capabilities which results into a possible application in drug delivery and management of the Parkinson's disease.

ACKNOWLEDGMENT

The SRF fellowship was provided by the Indian Council of Medical Research (ICMR) [Award No. 45/6/2019/MP/BMS], which is gratefully acknowledged by S. S. Suryawanshi. J P Jadhav thanks the Department of Biotechnology (DBT), Government of India for "The Interdisciplinary Programme of Life Sciences for Advanced Research and Education (IPLS- Reference No: BT/PR4572/INF/22/147/2012)". V A Bapat thanks Indian National Science Academy, New Delhi for honorary scientist fellowship. The authors are also thankful to Dr. S Otari, Dr. C B Aware and Dr. D D Andhare for their valuable suggestions.

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