

**Evaluation of antimicrobial activities and cell membrane permeability
of Iron oxide nanoparticles conjugated with phytocompounds of
Azadirachta indica.**

Submitted in partial fulfillment of the requirements for the award of a

Bachelor of Science in Biotechnology

By

AADRITA GUPTA - 41770001



**SCHOOL OF BIO AND CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

CATEGORY-1 UNIVERSITY BY UGC

Accredited with Grade "A++" by NAAC | 12B Status by UGC | Approved by AICTE
JEPPIAR NAGAR, RAJIV GANDHI SALAI, CHENNAI-600-119

APRIL-2024



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited "A++" Grade by NAAC | 12B Status by UGC | Approved by AICTE
www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of Aadrita Gupta (41770001) who carried out the project entitled " Evaluation of Antimicrobial Activities and Cell Membrane Permeability of Iron Oxide Nanoparticles Conjugated with Phytocompounds of *Azadirachta indica*" under my supervision from October 2023 to April 2024.

Internal Guide
Dr. SAQIB HASSAN

External Guide
Dr. KUNAL BISWAS

Head of the Department

Dr. V. RAMESH KUMAR(M.Phil., PhD)

Submitted for Viva-voce Examination held on 26 April 2024.

Internal Examiner

External Examiner

DECLARATION

I, **Aadrita Gupta** hereby declare that the Project Report entitled “ **Evaluation of Antimicrobial Activities and Cell Membrane Permeability of Iron Oxide Nanoparticles Conjugated with Phytocompounds of *Azadirachta indica***” done by me under the guidance of **Dr.Saqib Hassan**, Assistant Professor, Department of Biotechnology (Internal guide) and **Dr. Kunal Biswas**, Assistant Professor, IRC (External guide) at **Sathyabama Institute of Science and Technology** is submitted in partial fulfillment of the requirements for the award of a **Bachelor of Science Degree in Biotechnology**.

DATE: 27.04.2024



PLACE: CHENNAI

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **The Board of Management of Sathyabama Institute of Science and Technology** for their encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr.REKHA CHAKRAVARTHI, Dean, School of Science and Humanities**, and **Dr. V.RAMESH KUMAR, Head of the Department, Department of Biotechnology** for providing me with the necessary support during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr.Saqib Hassan**(Assistant Professor) and **Dr.Kunal Biswas**(Assistant Professor-Research, IRC) for their valuable guidance, suggestions, and constant encouragement paved the way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT

Nimbolide, the lead active compound is obtained from *Azadirachta indica* (Neem) leaves. It promises to show the highest levels of antimicrobial activities among all other compounds extracted from neem leaves. Further exploration of these neem compounds led to the descriptive study on nimbolide which shows the maximum levels of antimicrobial activities along with anticancer and antioxidant properties. Nanoparticles are always best known for their biological mobility. Its' encapsulating capacity profoundly binds the drug inside it and directly carries it to the target site for the treatment at the core level rather than surface manifestations. Superparamagnetic Nanoparticles are conjugated to aim an efficient drug delivery at target sites enhancing the quality of the phytocompound by providing a deeper penetration capacity. Iron oxide nanoparticle is chosen for the same which is commercially a very well-known conjugating agent with drugs in most of the pharmacological and nanobiotechnology fields. The biochemical testing was done using the disc diffusion method for both nimbolide and nimbolide-nanoparticle conjugated samples. *Escherichia coli* being a gram-negative species is chosen for the biochemical testing as they have a cell membrane that can easily be permeable for substances or drugs. They were environmental isolates that were subjected to various confirmatory tests. The zone of inhibition at different MICs would indicate the efficacy and resistance of the drug against the pathogen.

Keywords: Nimbolide, iron oxide nanoparticle, *E.Coli*, disc diffusion, zone of inhibition.

TABLE OF CONTENTS:

S.No	CONTENTS	PAGE NO.
	ABSTRACT	V
	LIST OF ABBREVIATIONS	VII
	LIST OF FIGURES	VIII
	LIST OF TABLES	IX
1	INTRODUCTION	1
	1.1 ANTIMICROBIAL PROPERTIES OF NIMBOLIDE	2
	1.2 NANOPARTICLES AND THEIR SCOPE	2
	1.3 APPLICATIONS OF NANOPARTICLES IN DIFFERENT FIELDS	3
2	LITERATURE REVIEW	6
3	AIM AND SCOPE	11
4	MATERIALS AND APPARATUS	12
	4.1 MATERIALS REQUIRED	12
	4.2 APPARATUS REQUIRED	14
5	METHODOLOGY	16
	5.1 HPLC ANALYSIS	16
	5.2 UV CHARACTERIZATION OF THE NIMBOLIDE	17
	5.3 FTIR ANALYSIS OF THE NIMBOLIDE	19
	5.4 IRON OXIDE NANOPARTICLE	20
	5.4.1 CONJUGATION USING SONICATION	21
	5.5 UV CHARACTERIZATION OF THE CONJUGATED SAMPLE	22
	5.6 FTIR ANALYSIS OF THE CONJUGATED SAMPLE	22
	5.7 GRAM STAINING	23
	5.7.1 SLIDE PREPARATION	24
	5.8 DISC DIFFUSION METHOD	25
6	RESULTS AND DISCUSSION	28
	6.1 DISC DIFFUSION RESULTS	28
	6.2 FESEM ANALYSIS RESULTS	31
7	SUMMARY AND CONCLUSION	33
	REFERENCES	35

LIST OF ABBREVIATIONS

MIC	Minimum Inhibitory Concentration
MRI	Magnetic Resonance Imaging
CT	Computed Tomography
MAE	Microwave-Assisted Extraction
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
UV	Ultra-Violet
FTIR	Fourier Transform Infrared
FESEM	Field Emission Scanning Electron Microscope
L.B	Lysogeny Broth
<i>E. Coli</i>	<i>Escherichia Coli</i>
μl	Microlitre
nm	Nanometre

LIST OF FIGURES

Figure no.	Figure Name	Page no.
1.1	Neem tree and its different compounds	1
1.2	2D and 3D structures of the Nimbolide	2
4.1	Flowchart of the entire process	12
5.1	HPLC Analysis Report	17
5.2	UV-Vis Spectroscopy	19
5.3	FTIR Characterization Report	20
5.4	Nimbolide conjugated to the nanoparticle	21
5.5	UV spectroscopy of the conjugated sample	22
5.6	FTIR Analysis Report	23
5.7	Prepared slide for staining	25
5.8	Visualization of the slide at 40X	25
5.9	L.B Broth inoculated with <i>E.Coli</i> stain	26
6.1	Disc diffusion of pure nimbolide sample at highest MIC	28
6.2	Disc diffusion of pure nimbolide at lower MICs	28
6.3	Disc diffusion of nanoparticle conjugated sample at higher MIC	29
6.4	Disc diffusion of nanoparticle conjugated sample at lower MICs	29
6.5	MIC vs Zone of Inhibition-comparative Evaluation	31
6.6	FESEM Image of the conjugate sample	31

LIST OF TABLES

TABLE NO.	TABLE NAME	PAGE NO.
5.1	L.B broth Preparation	26
5.2	Mueller Hilton Agar	26
6.1	Zone of Inhibition at Different MICs	30

CHAPTER1: INTRODUCTION

This project redefines the antimicrobial activities of traditional plant neem (*Azadirachta indica*) focusing on a particular type of phytochemical. The neem tree belongs to the mahogany family of Meliaceae and is scientifically known as *Azadirachta indica*. The availability of neem plants is huge in the sub-tropical areas of India, Pakistan, Bangladesh, and Nepal. A neem tree can grow up to 15 -20 meters in height though few can reach up to 40 meters. They have dense, spreading foliage with small, compound leaves that are arranged alternately on the branches. The tree produces white flowers with a sweet scent and small, oval-shaped fruits that turn yellow when ripe. There are various beneficial phytochemicals obtained from neem either from its leaves, stem, bark, roots, etc. [Subapriya *et al.*, 2005].

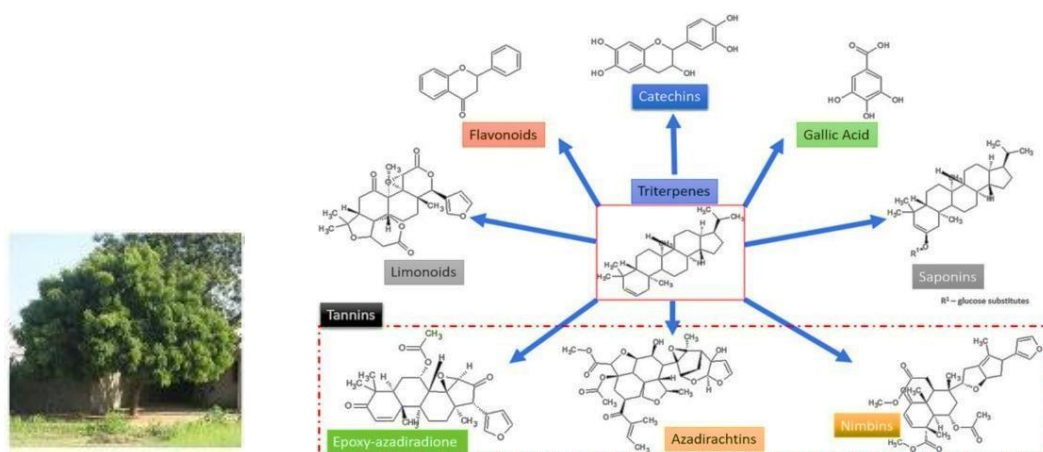


Fig.1.1: Neem tree and its different compounds [Islas *et al.*, 2020]

The phytochemicals present in neem are terpenoids and flavonoids, which act as reducing agents as well as capping agents and help in the stabilization of the nanoparticles. The main point to be stressed was on the extraction of the most active compound from neem which shows comparatively increased antimicrobial characteristics among all the other compounds of neem. Out of all the phytochemicals explored to date, the lead phytochemical with increased antimicrobial properties is the nimbolide. Nimbolide is a phytochemical obtained from pure neem leaf extracts following a series of procedures. Nimbolide has been used in traditional Indian medicine to treat various diseases such as infections,

gastric ulcers, cancers, etc. Along with antimicrobial characteristics, nimbolide also possesses anticancer and antioxidant properties [Ragini *et al.*, 2021].

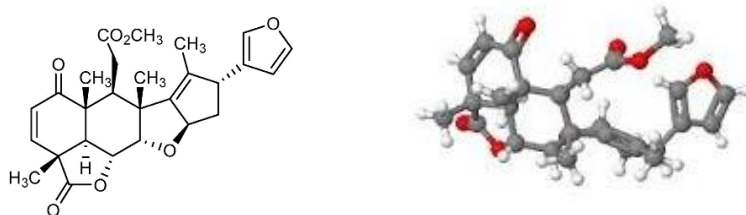


Fig.1.2: 2D and 3D structures of the nimbolide [Suttiaporn *et al.*, 2020]

1.1: ANTIMICROBIAL PROPERTIES OF NIMBOLIDE:

Nimbolide is a bioactive compound found in the neem tree (*Azadirachta indica*). It has garnered interest in scientific research due to its potential medicinal properties, including antimicrobial activity.

Nimbolide has demonstrated significant antibacterial activity against a wide range of bacterial strains, both Gram-positive and Gram-negative bacteria. Studies have shown that it can inhibit the growth of bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. Nimbolide's mechanism of action against bacteria may involve disrupting bacterial cell membranes, interfering with essential metabolic pathways, or inhibiting bacterial enzymes.

Neem's antimicrobial characteristic stems from its rich composition of bioactive compounds, including nimbin, nimbidin, azadirachtin, and other limonoids. These compounds exhibit potent antibacterial, antiviral, antifungal, and antiparasitic activities, making neem a versatile natural remedy for various infections [Sarkar *et al.*, 2021]

1.2. NANOPARTICLES AND THEIR SCOPE:

Nanoparticles, being an emerging aspect eventually gained importance in every field of science. They play a vital role in nanotechnology streams, biotechnological studies, pharmacology, etc. Nanobiotechnology is a field of science that integrates nanoscience along with biological systems associated with different nanomedicines. Nanoparticles are small spherical polymeric substances whose size ranges around

10⁻⁹ nanometers. Nanomaterials-based formulations have made a huge impact on drug delivery systems. They have been a preferable choice for drug delivery because of their small size, large surface area, increased solubility, and increased bioavailability. The main aim of using nanoparticles for drug delivery is because of their greater penetration through the cell lines at their specific target sites [Heiligtag *et al.*,2013]

Magnetic nanoparticles are the ones consisting of magnetic elements, like nickel, cobalt, chromium, manganese, gadolinium, and their chemical compounds. Magnetic nanoparticles are superparamagnetic because of their nanoscale size, offering great potential in a variety of applications either in bare form or surface-coated form. Especially, ferrite nanoparticles (Iron oxide) are the most explored magnetic nanoparticles to date, which can be enhanced by clustering several other individual superparamagnetic nanoparticles into clusters to form magnetic beads. [Hasani *et al.*,2013].

Magnetic nanoparticles can be selectively attached to different functional molecules and allow transportation to a targeted location under an external magnetic field from an electromagnet or permanent magnet. To prevent aggregation and minimize the interaction of the particles with the system environment, surface coating may be required. The surface of ferrite nanoparticles is often modified by surfactants, silica, silicones, or phosphoric acid derivatives to increase their stability in solution. In general, coated magnetic nanoparticles have been widely used in several medical applications, such as cell isolation, immunoassay, diagnostic testing, and drug delivery [Latham *et al.*, 2008].

The scope of nanoparticles is vast and encompasses various fields, including medicine, electronics, environmental science, and materials science.

1.3. APPLICATIONS OF NANOPARTICLES IN DIFFERENT FIELDS:

Biomedical Applications: Nanoparticles hold immense promise in medicine, particularly in drug delivery, imaging, and therapy. They can be modified to encapsulate drugs and right away deliver them to specific targets in the body, minimizing side effects and improving therapeutic efficacy. Additionally,

nanoparticles are also used as a contrast agent in medical imaging techniques like magnetic resonance imaging (MRI) and computed tomography (CT) scans [Parveen et al.,2017]

Cancer Therapy: Nanoparticles are revolutionizing cancer treatment through targeted drug delivery and photothermal therapy. By functionalizing nanoparticles with targeting ligands, they can selectively accumulate in tumor tissues, enhancing drug uptake and minimizing damage to healthy cells. Moreover, certain nanoparticles exhibit photothermal properties, allowing them to destroy cancer cells when exposed to light selectively [Gavas *et al.*, 2021]

Environmental Remediation: Nanoparticles play a crucial role in environmental science by facilitating pollution control and remediation. They can be utilized for wastewater treatment, air purification, and soil remediation. Nanoparticles such as titanium dioxide (TiO₂) are photocatalysts that degrade organic pollutants and microbial contaminants under sunlight, offering a sustainable solution for environmental cleanup.

Materials Science: Nanoparticles are utilized to enhance the properties of materials in various industries. In nanocomposites, nanoparticles are dispersed within a matrix to improve mechanical strength, thermal stability, and electrical conductivity. Nanoparticles also enable the development of lightweight and durable materials for aerospace, automotive, and construction applications [Hiremath et al., 2021]

Energy Storage and Conversion: Nanostructured materials are currently of interest for such devices because of their high surface area, novel size effects, and significantly enhanced kinetics. Nanoparticles play a crucial role in energy storage and conversion technologies such as batteries, fuel cells, and solar cells. Nanomaterials, such as graphene and carbon nanotubes, offer high surface area and conductivity, enhancing the performance of electrodes in energy storage devices. Moreover, nanoparticles are used as catalysts in fuel cells and as light-absorbing materials in photovoltaic cells for efficient energy conversion [Guo et al., 2008]

By functionalizing nanoparticles with targeting ligands, such as antibodies or peptides, researchers can enhance their specificity to diseased tissues, minimizing off-target effects and improving therapeutic outcomes. Many drugs usually suffer

from poor stability which limits their bioavailability as well as clinical efficacy. To overcome these challenges nanoparticles have been empowered in such a way that they can encapsulate these drugs protecting them from degradation. This augments the solubility of the drug resulting in better therapeutic outcomes. The release of drugs by nanoparticles can be controlled and altered depending on the different target sites, pH, and temperature.

Nanoparticles have also shown their efficacy in vaccine formulations. When nanoparticles are conjugated with vaccines it tends to enhance the antigen presentation, immunogenicity, and vaccine stability. These conjugated formulations have gained importance in the recent past for various treatments including severe infectious diseases and cancer [D'Amico et al.,2021].

CHAPTER 2- LITERATURE REVIEW

Previously Nimbolide and many such lead phytocompounds have been unexplored until the chemical-based formulations of medicines turned out to be harmful in the longer run. Traditional medicinal plants like neem and tulsi are well known for their antibacterial effects and have been used for various conventional treatments for a very long period.

Nimbolide from *Azadirachta indica* and its derivatives plus first-generation cephalosporin antibiotics: a novel drug combination for wound-infecting pathogens by [Dhanya et al., 2015] was another approach towards the exploration of the *In vitro* efficacy of nimbolide with cephalosporin against infectious pathogens.

In vitro, the comparative cytotoxic effect of Nimbolide: A limonoid from *Azadirachta indica* (Neem tree) on cancer cell lines and normal cell lines through an MTT assay by [Kashif et al., 2017] was performed for the cytotoxicity analysis.

Azadirachta indica: Antibacterial activity of neem against different strains of bacteria and their active constituents as preventive in various diseases by [Herrera-Calderon et al., 2019] mainly aimed to focus on the antifungal, antibacterial, antiviral activities of neem and its constituent compounds and the utilization of their medicinal properties.

The rapid process of extraction and purification of the nimbolide from the leaves of the neem tree through microwave-assisted extraction (MAE) coupled with a chromatographic technique was accomplished. The crude with a maximum content of nimbolide could be recovered from neem leaves through MAE [Suttiaporn et al., 2020].

Nimbolide, a neem limonoid, is a promising candidate for the anticancer drug arsenal by [Nagini et al., 2021] who redefined the anticancer traits and properties of nimbolide.

Nimbolide is reported to exert potent antiproliferative effects on myriad cancer cell lines and chemotherapeutic efficacy in preclinical animal tumor models. The

potentiality of nimbolide to circumvent multidrug resistance and aid in targeted protein degradation broadens its utility in enhancing therapeutic modalities and outcomes. It has been observed that nimbolide prevents proliferation, apoptosis evasion, invasion, angiogenesis, metastasis, and inflammation.

In vitro, antibacterial activity of nimbolide was done against *Helicobacter pylori* by [Marina *et al.*,2022] to verify the post-antibiotic effects of nimbolide. The increasing demand for novel therapeutic drugs from phytocompounds naturally instead of chemical means led to these experiments and explorations of medicines. The lack of hemolytic activity, synergistic activity at low pH, and bactericidal properties even against bacteria in a state of growth arrest are all ideal pharmacological and biologically relevant properties for a potential new agent.

Molecular docking of nimbolide extracted from leaves of *Azadirachta indica* with protein targets to confirm the antifungal, antibacterial, and insecticidal activity by [Navinraj *et al.*,2023] mainly focused on the compound isolated from the leaves of *Azadirachta indica*, which was screened both *in vitro* and *In silico* for its antimicrobial activity against *oryzae pv. Oryza. oryzae* forms an inhibition zone of about 20.20 mm, and *P.xylostella* showed about 66.66% mortality against nimbolide.

A brief overview of the complexity of cancer tumor's microenvironment is presented, followed by discussion of combinatorial therapies categorized based on chemotherapeutic agents, nucleic acid or therapeutic proteins, energy-based therapies and imaging techniques for theranostic application. Different nanotechnological platforms are developed for combination therapy as tools with a great potential to tackle the most critical issues of current cancer treatments by [Shrestha *et al.*, 2019]. A deeper understanding of these nanotechnologies and their possible long-term effects on biological systems is needed for further clinical translation.

A nanoparticle has emerged as a promising strategy for the efficient delivery of drugs used for the treatment of some diseases by specific targeting. These carriers are designed in such a way that they are independent in the environments and selective at the pharmacological site. The formation of nanoparticle and physiochemical

parameters such as pH, monomer concentration, ionic strength as well as surface charge, particle size and molecular weight are important for drug delivery. Further, these nanoparticles have the capability to reverse multidrug resistance a major problem in chemotherapy. Well-established therapies commonly employed in cancer treatment include surgery, Chemotherapy, immunotherapy, and radiotherapy. This mini-review by [Sivasankar *et al.*,2010] is summarizing the information on different methods of manufacturing nanoparticles, focusing on its properties and biological transport.

Synthesized drug conjugates can be readily encapsulated into a lipid-coated polymeric drug-delivery nanoparticle, which significantly improves the cytotoxicity of the drug conjugates as compared to the free drug conjugates. So, a new approach to loading multiple drugs onto the same drug-delivery nanocarrier in a precisely controllable manner, by covalently preconjugating multiple therapeutic agents through hydrolyzable linkers to form drug conjugates, is reported by [Aryal *et al.*, 2010].

This Minireview by [Liu *et al.*, 2020] presents an overview of recent research on developing nanoparticles with high drug loading (>10 wt%) from the perspective of synthesis strategies, including post-loading, co-loading, and pre-loading. Based on these three different strategies, various nanoparticle systems with different materials and drugs are summarized, and the advantages and disadvantages of these strategies are discussed to provide useful design rules for the future development of high-drug-loading nanoparticles.

Targeting specific sites *in vivo* for the delivery of therapeutic compounds presents a major obstacle to the treatment of many diseases. One targeted delivery technique that has gained prominence in recent years is the use of magnetic nanoparticles. In these systems, therapeutic compounds are attached to biocompatible magnetic nanoparticles and magnetic fields generated outside the body are focused on specific targets *in vivo*. The fields capture the particle complex resulting in enhanced delivery to the target site. This review by [Dobson *et al.* ,2006] focuses on technical aspects of magnetic targeting as well as nanoparticle design and animal and clinical trials.

This review by [Rassu *et al.*, 2023] basically summarises strategies for enhancing the central nervous system's phytochemical efficacy. Particular attention has been paid to the use of phytochemicals in combination with other drugs (co-administrations) or administration of phytochemicals as prodrugs or conjugates, particularly when these approaches are supported by nanotechnologies exploiting conjugation strategies with appropriate targeting molecules.

Improvement of biological activities of medicinal plants in combination with metal nanoparticles is highly appealing. Here, in this review by [Patra *et al.*, 2018] bio-conjugated AuNP and AgNP have been synthesized at room temperature using leaf extract of *Saraca asoca* a medicinal plant. Phytochemicals present in the aqueous leaf extract of *Saraca asoca*, are acting as reducing, capping and stabilizing agent. The formation of nanoparticles is confirmed and characterized by different instrumental techniques like UV-visible and IR spectroscopy, SEM, TEM and XRD analysis.

Magnetic nanoparticles (MNPs), particularly made of iron oxides, have been extensively studied as diagnostic imaging agents and therapeutic delivery vehicles. This review by [Boubbou *et al.*, 2018] specially emphasises on the 'recent advancements of drug-conjugated MNPs used for therapeutic applications'. The most prevalent preparation methods and chemical functionalization strategies required for translational biomedical nanoformulations are outlined. Particular attention is, then, devoted to the tailored conjugation of drugs to the MNP carrier according to either noncovalent or covalent attachments, with advantages and drawbacks of both pathways conferred.

Novel methods are necessary to reduce morbidity and mortality of patients suffering from infections with *Pseudomonas aeruginosa*. Being the most common infectious species of the *Pseudomonas* genus, *P. aeruginosa* is the primary Gram-negative etiology responsible for nosocomial infections. Due to the ubiquity and high adaptability of these species, an effective universal treatment method for *P. aeruginosa* infection still eludes investigators, despite the extensive research in this area and looks for better antimicrobial or antibacterial activities by [Armijo *et al.*, 2020].

Medical applications and biotechnological advances, including magnetic resonance imaging, cell separation and detection, tissue repair, magnetic hyperthermia and drug delivery, have strongly benefited from employing iron oxide nanoparticles (IONPs) due to their remarkable properties, such as superparamagnetism, size and the possibility of receiving a biocompatible coating. Ongoing research efforts focus on reducing drug concentration, toxicity, and other side effects, while increasing efficacy of IONPs-based treatments. This review by [Arias et al., 2018] highlights the methods of synthesis and presents the most recent reports in the literature regarding advances in drug delivery using IONPs-based systems, as well as their antimicrobial activity against different microorganisms.

Investigating the interaction patterns at nano-bio interface is a key challenge for the safe use of nanoparticles (NPs) in any biological system. This study intends by [Arakha et al., 2015] to explore the role of interaction patterns at the iron oxide nanoparticle (IONP)-bacteria interface affecting the antimicrobial propensity of IONP. The comparative data from Fourier transform infrared spectroscopy, XRD and zeta potential analyzer indicated the successful coating of IONP surface with chitosan molecule. Additionally, the nanocrystals obtained were found to have a spherical size with 10–20 nm diameter. The BacLight fluorescence assay, bacterial growth kinetic and colony-forming unit studies indicated that n-IONP (<50 µM) has insignificant antimicrobial activity against *Bacillus subtilis* and *Escherichia coli*.

Pulsed laser ablation of iron rod targets in water-based solutions was carried out, varying the ablation parameters. The structural, morphological and compositional characteristics of the nanoparticles were studied by micro-Raman, dynamic light scattering (DLS), X-ray photoelectron (XPS) spectroscopies and electron scanning microscopy (SEM/STEM). Slight changes in the ablation parameters result in significant variations in the nanoparticles morphology. The antimicrobial activity of iron oxide nanoparticles on *Staphylococcus aureus* was studied using of MTT assay by [Fazio et al., 2016] The results indicate that the iron oxide nanoparticles are interesting for potential applications as a vector for drug delivery and a constituent of specific platforms for drug targeting.

CHAPTER 3: AIM AND SCOPE

This project aims to identify and redefine the antimicrobial characteristics of traditional plant neem when conjugated with magnetic nanomaterial (Iron oxide) and to analyze the efficacy of the final product obtained.

- To extract pure neem powder from the dried neem leaves for the maceration process.
- To perform soxhlation or microwave-assisted extraction (MAE) where ethanol is used as a solvent for better solubility than in water.
- To perform RP-HPLC to analyze our desired phytocompound's presence and availability in the given sample.
- To perform UV and FTIR characterization for identification and characterization of the various analytes in the crude sample.
- To subject the sample to conjugation along with iron oxide nanoparticles in a probe sonicator for efficient delivery at target locations, after all the sample testing procedures.
- To test the final sample against a gram-negative microorganism, an environmental isolate of *E. coli* sps, to check the antimicrobial activity using the disc diffusion method.
- To analyze 3 different cases using the disc diffusion method on Mueller Hilton agar.
- Case 1: Nimbolide tested on *E.coli* sps
- Case 2: Nimbolide conjugated with iron oxide and tested on *E.coli* sps.
- Case 3: The control (only *E.coli* grown agar)

CHAPTER 4: MATERIALS AND APPARATUS

The approach towards the extraction of nimbolide from the dried neem leaf powder included a series of procedures. The first step included natural drying of the neem leaves in the absence of sunlight under normal conditions. The neem leaf powder can be subjected to soxhlation or using conventional methods to carry out the process of soxhlation.

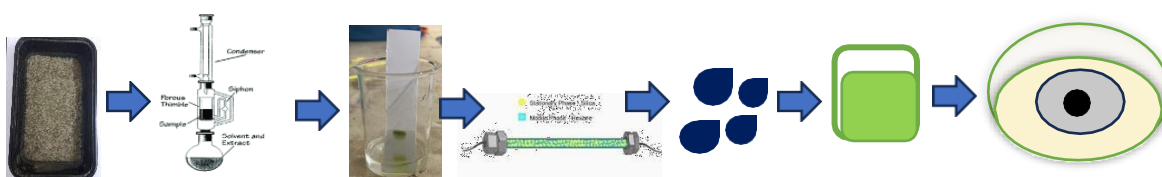


Fig.4.1: Flowchart of the entire process

4.1 MATERIALS REQUIRED:

- Neem leaf powder

Neem leaves are finely powdered neem leaf powder is derived from the leaves of the neem tree (*Azadirachta indica*) and is widely used for its various health benefits. The leaves of the neem tree are dried and ground into a fine powder, which can then be used in various ways.

- Ethanol as a solvent for better solubility

Ethanol, also known as ethyl alcohol, is a commonly used solvent in various industries due to its versatility, effectiveness, and relatively low toxicity. Ethanol is a polar solvent, meaning it can dissolve both polar and non-polar substances. It's miscible with water and can dissolve a wide range of organic compounds, including many pharmaceuticals, Flavors, and fragrances.

- Whatman filter paper

Whatman filter paper is a type of filtration medium used in laboratories and various industrial applications to separate solids from liquids or to collect solid particles from a liquid suspension. Whatman is a brand name known for producing high-quality filter papers, which are manufactured by Cytiva (formerly part of GE Healthcare Life Sciences).

Whatman filter papers are available in various pore sizes, ranging from coarse to fine, depending on the application requirements

- L.B broth

LB broth, or Lysogeny Broth, is a nutrient-rich medium commonly used in microbiology laboratories for the growth and propagation of bacteria. It was developed by scientists Bertani and Weigle in 1953. LB broth is a liquid medium that supports the growth of a wide variety of bacteria, making it a versatile choice for bacterial culture.

- *E.coli* strain (environmental isolate)

Escherichia coli or *E.coli* is a Gram-negative species. It is a rod-shaped bacterium that is mainly found in the intestines of all warm-blooded animals, including humans. Though some strains of *E. coli* are harmless and even beneficial, others can cause illness, particularly gastrointestinal infections.

- Nanoparticle- Iron oxide

Iron oxide nanoparticles primarily consist of iron and oxygen atoms. Magnetite, Hematite, and Maghemite are the constituents of this sample.

- Crystal violet

Crystal violet is a synthetic dye with the chemical formula $C_{25}H_{30}ClN_3$. It belongs to the class of triaryl methane dyes and is commonly used as a biological stain in microscopy and bacteriology.

- Decolourizer

In laboratory staining techniques, a decolorizer is a chemical agent used to remove or decolorize stains from certain types of cells or tissues, particularly in the Gram staining process. In Gram staining, the decolorizer plays a crucial role in washing away or removing the excess stain from the cells.

- Gram's Iodine

Gram's iodine, also known as Gram's iodine solution or Gram iodine, is a chemical reagent used in the Gram staining technique, a widely employed method in microbiology to differentiate between Gram-positive and Gram-negative bacteria. Gram's iodine solution plays a critical role in the staining process by forming a complex with crystal violet, one of the primary stains that is used in the procedure.

- Safranin

Safranin is a biological dye commonly used in histology and microbiology for staining purposes. It belongs to the family of basic or cationic dyes and is often employed in combination with other stains like crystal violet in staining techniques like Gram staining.

- Immersion oil

Immersion oil is a specialized oil used in microscopy to improve the resolution and clarity of images observed under high-power objectives, particularly in oil immersion microscopy.

- Heat-fixed Slides

Heat-fixed slides are prepared microscope slides that have been subjected to the process of heat fixation after the strain is smeared onto them. This process is commonly used in microbiology and histology to affix microorganisms or tissue samples to the slide, making them suitable for staining and microscopic examination.

- Mueller Hilton Agar

Mueller-Hinton agar is a type of growth medium used for antimicrobial susceptibility testing, particularly for bacteria. They have high diffusion rates which makes it more convenient for biochemical testing procedures in laboratories.

4.2 APPARATUS REQUIRED:

- Shaker incubator

A shaker incubator is a laboratory instrument used for the agitation and incubation of samples under controlled temperature conditions. It combines the functions of a shaker and an incubator, providing gentle agitation to promote mixing or dissolution of samples while maintaining a stable temperature environment.

- Probe ultra sonicator

A probe sonicator, also known as an ultrasonic homogenizer or ultrasonic disruptor, is a laboratory instrument used for disrupting, homogenizing, emulsifying, or dispersing samples through the application of high-frequency sound waves. It is a versatile tool used in various fields such as biology, chemistry, materials science, and nanotechnology.

- UV spectroscopy

UV characterization refers to the use of ultraviolet (UV) spectroscopy techniques to analyze and characterize molecules based on their interaction with UV light. UV spectroscopy involves measuring the absorbance or transmission of UV light by a sample across a range of wavelengths, typically in the ultraviolet region of the electromagnetic spectrum (200 to 400 nanometres).

- FTIR

FTIR (Fourier Transform Infrared) characterization refers to the use of FTIR spectroscopy techniques to analyze and characterize molecules based on their interaction with infrared light. FTIR spectroscopy involves measuring the absorption of infrared radiation by a sample across a range of wavelengths ranging from 200nm.

- HPLC

HPLC (High-Performance Liquid Chromatography) is used for a variety of reasons across numerous industries and scientific disciplines due to its versatility, sensitivity, and precision. HPLC allows for the separation of complex mixtures of compounds into individual components based on their chemical properties such as size, charge, polarity, and affinity for the stationary phase. Provides a quantitative analysis for the samples.

- FESEM

FESEM (Field Emission Scanning Electron Microscopy) is performed for a variety of reasons across scientific research, materials science, engineering, and various industries due to its unique capabilities. FESEM provides high-resolution imaging capabilities, allowing for the visualization of surface morphology, microstructure, and nanoscale features of materials with exceptional detail. This is essential for studying the surface topography of materials at the micro and nanoscale. It shows the binding of our samples with other analytes present in it. Here FESEM is used to analyze the binding of our phytochemical along with our desired nanoparticle. It is used to check and verify whether the nanoparticle has affected the structure of the nimbolide after sonication.

CHAPTER-5 METHODOLOGY

Neem powder is taken around 3.9g in 100 ml of the solvent(ethanol) in a conical flask and macerated well in a shaker incubator for about 24 to 48 hrs under room temperature. The macerated mixture was then filtered out using Whatman filter paper into another flask and then it was poured into properly autoclaved and sterilized petri plates. These plates were covered with aluminum sheets or foils and medium-sized holes were made for the solvent to evaporate out and leave us with the crude sample. The plates were left as it is for about 2 to 3 days for them to dry out entirely. The dried samples were then scrapped out by adding 2-3 drops of the same solvent used for the process and then stored the samples in Eppendorf's tubes.

5.1. HPLC ANALYSIS

High-Performance Liquid Chromatography (HPLC) is a chromatographic technique that is used to separate, identify, and quantify components in a mixture. It's widely used in analytical chemistry and biochemistry for various applications, also used in pharmaceuticals, food and beverage analysis, environmental monitoring, and more.

HPLC works on the same basic principles as other chromatographic techniques but uses a liquid mobile phase instead of a gas. The components of the sample are dissolved in a liquid solvent and pumped through a column that is packed with a stationary phase. As the sample passes through the column, different components tend to interact differently with the stationary phase, which causes them to separate based on their different chemical properties and so on.

HPLC facilitates the identification of analytes present in a sample by comparing their retention times with those of known standards or reference compounds. The characteristic retention time of each analyte allows for its unambiguous identification, enabling researchers to determine the composition of a mixture and quantify the concentration of individual components.

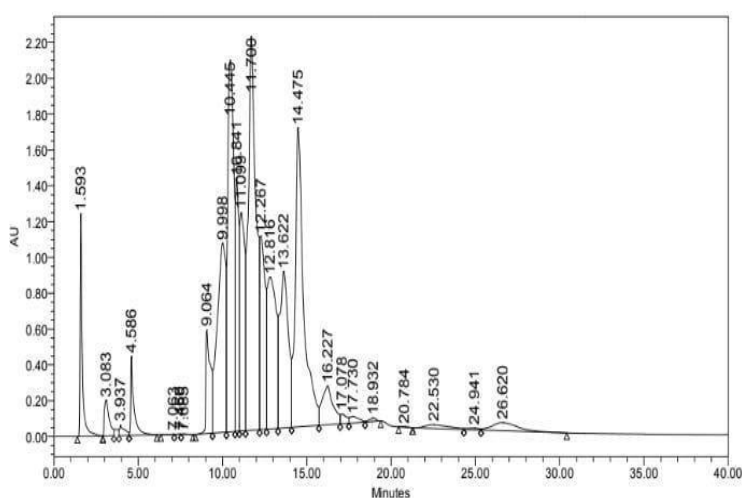
HPLC was performed for the samples, Nimbolide was identified as the one with retention time around 2.8-3.2 when cast under the wavelength of range 200–300nm. Nimbolide was found at 3.08RT. So, nimbolide was found in the given analysed sample and the presence was noted accordingly.



Default Individual Report

SAMPLE INFORMATION

Sample Name:	A Indica	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	
Vial:	1:A2	Acq. Method Set:	Ar tumerone 10 to 100 H2O ACN
Injection #:	4	Processing Method:	A Indica
Injection Volume:	20.00 ul	Channel Name:	213.0nm
Run Time:	40.0 Minutes	Proc. Chnl. Descr.:	PDA 213.0 nm
Date Acquired:	27-Jan-10 2:05:54 PM IST		
Date Processed:	27-Jan-10 11:56:20 PM IST		



	RT	Area	% Area	Height
9	9.998	37394116	9.24	1055714
10	10.445	51717925	12.78	2073332
11	10.841	19648301	4.85	1410587
12	11.099	24798126	6.13	1219103
13	11.700	72349452	17.87	2198227
14	12.267	23738094	5.86	1079732
15	12.816	31415814	7.76	846521
16	13.622	31385153	7.75	874134
17	14.475	58335230	14.41	1671681
18	16.227	9877360	2.44	215792
19	17.078	1363858	0.34	53351
20	17.730	1359881	0.34	36720
21	18.932	515665	0.13	18858
22	20.784	84401	0.02	3321
23	22.530	2080266	0.51	20007
24	24.941	497681	0.12	9950
25	26.620	4920957	1.22	45889

	RT	Area	% Area	Height
1	1.593	11292334	2.79	1243511
2	3.083	3968738	0.98	200660
3	3.937	1206862	0.30	58906
4	4.586	5609612	1.39	442666
5	7.063	62423	0.02	2752
6	7.460	65751	0.02	2975
7	7.685	70082	0.02	2894
8	9.064	11066514	2.73	577562

Fig.5.1: HPLC Analysis Report

5.2 UV CHARACTERIZATION OF THE NIMBOLIDE

Then for further characterization, the sample was subjected to UV spectroscopy analysis. UV spectroscopy is a quantitative analysis or determination of the various constituents or analytes present in a given sample. Absorption spectroscopy is a quantitative chemical analysis method that is based on the Beer-Lambert Law. This law states that the absorption of visible light passing through a certain substance is directly proportional to the concentration of that substance. In short, the more concentrated a substance is, the more of the light it will absorb. A detailed descriptive study of the crude sample is necessary for a research report to depict accurate data. This type of spectroscopy is known as UV-visible spectroscopy

because the electromagnetic radiation wavelengths at which it operates usually range from 200nm to around 1,000nm.

With the electromagnetic spectrum in mind, this means that UV-visible spectroscopy includes the entire range of ultraviolet waves, which have a length of 200-400nm, as well as the entire visible light range, which spans from 390 to 700 nm.

Quantitative Analysis: UV spectroscopy is commonly used for the quantitative analysis of compounds in a solution. It is very useful for determining concentrations of substances like proteins, nucleic acids, and drugs.

Qualitative Analysis: UV spectroscopy can identify functional groups and chemical structures based on characteristic absorption patterns. This is especially helpful in organic chemistry for compound identification.

Kinetic Studies: UV spectroscopy can be employed to study reaction kinetics by monitoring changes in absorbance over time. This is crucial for understanding reaction mechanisms and reaction rates.

Quality Control: In pharmaceuticals, UV spectroscopy is used for quality control purposes to ensure the purity and concentration of drugs in formulations.

Environmental Analysis: UV spectroscopy can be used in environmental analysis to measure the concentration of pollutants in water and air.

Protein and Nucleic Acid Analysis: UV spectroscopy is extensively used in biochemistry to analyze proteins and nucleic acids, such as DNA and RNA.

The wavelength approximately estimated for this analysis was 200-600nm where Nimbolide shows a peak at 213nm depicting that it lies between the 200-300nm wavelength range.

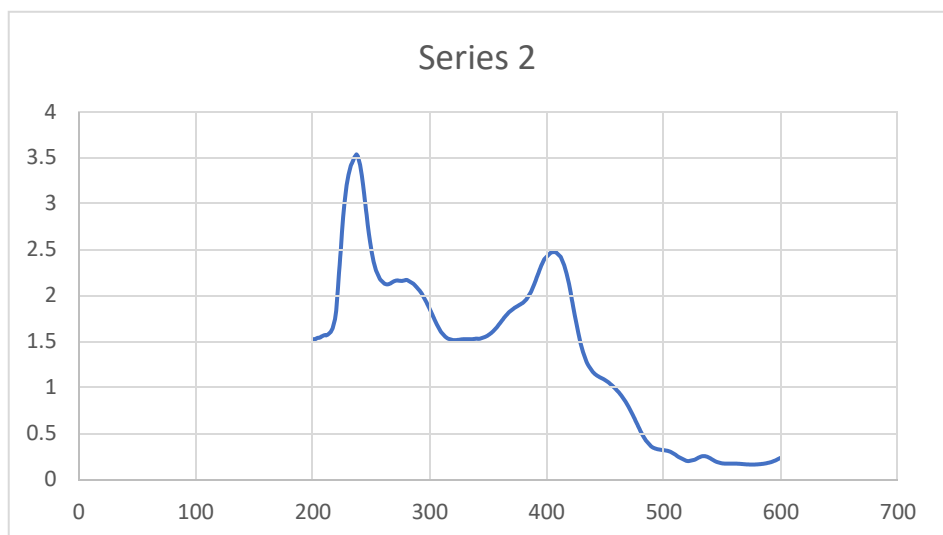


Fig.5.2: UV -Vis spectroscopy

5.3 FTIR ANALYSIS OF THE NIMBOLIDE

For a more detailed analysis of the constituents of the nimbolide sample, FTIR analysis is done to identify and characterize unknown materials like solids, liquids, powders, air, etc.

FTIR spectroscopy measures the absorption of infrared radiation by a sample. Molecules absorb specific frequencies of infrared light that correspond to the vibrational modes of the bonds within the molecule. By measuring these absorbance frequencies, FTIR spectroscopy provides information about the chemical composition, structure, and bonding of a sample.

The wavelengths selected for this sample analysis are 500-4500nm. The sample should be in a solution made with the used solvent for it to be used for proper FTIR analysis.

There were around 4000 or more compounds found within the crude sample. Since, the neem leaves were dried up entirely, powdered, and mixed with ethanol as a solvent the presence of water was not expected in this analysis. The analysis report showed the presence of water in it because leaves even after getting dried up still had moisture content in their epidermis. The sample contained water which lies in or around 2000 – 3000 and air which lies between 500 -1000 cm^{-1} . The presence of nimbolide was proved through this analysis.

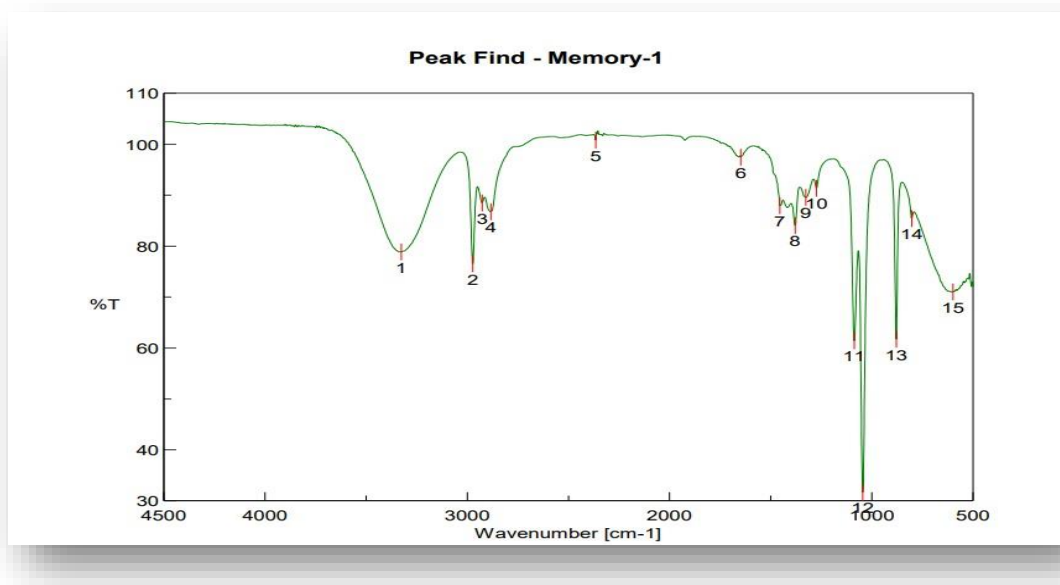


Fig.5.3: FTIR Characterization Report

5.4. IRON OXIDE NANOPARTICLE

Nanoparticles are microscopic particles with at least one dimension less than 100 nanometers. They can be composed of various materials, like metals, metal oxides, polymers, ceramics, and carbon-based materials. Nanoparticles demonstrate unique physical and chemical properties due to their small size, high surface area-to-volume ratio, and quantum effects.

Nanoparticles are increasingly utilized in drug delivery systems due to their unique properties that can improve the pharmacokinetics, therapeutic efficacy, and safety profiles of drugs.

Encapsulation:

Nanoparticles can encapsulate drug molecules within their matrix, improving their solubility, stability, and protection from degradation.

Hydrophobic Drug Delivery:

Hydrophobic drugs can be encapsulated within the hydrophobic core of nanoparticles, enhancing their solubility in aqueous environments.

Passive Targeting:

Nanoparticles can accumulate passively at the target site due to the enhanced permeability and retention (EPR) effect, particularly in tumors and inflamed tissues with leaky vasculature.

Active Targeting:

Surface functionalization of nanoparticles with ligands such as antibodies, peptides, or aptamers allows for specific binding to target cells or tissues, enhancing drug delivery to desired locations while minimizing off-target effects.

5.4.1 Conjugation using sonication

Iron oxide is the nanoparticle used in this process for conjugation with the target sample nimbolide. Iron oxide is chosen for this purpose because of its magnetic traits and behaviors. They have high magnetic perceptivity and superparamagnetic characteristics. Nanoparticles of magnetic iron oxide also include maghemite and magnetite, which are highly biocompatible and have low toxicity. A prior sample testing of iron oxide was performed, where the purity of the sample was identified as 99.9%. The average particle size is around 30-80nm with a specific surface area (SSA) around 60-90 m²/g. The bulk density of iron oxide was found to be 0.66g/cm³.

The iron oxide nanoparticle is conjugated with our target drug nimbolide using sonication. A probe Ultrasonicator is used for the conjugation process. The sample is run for 30 minutes, making sure both of them are in the form of liquid solution. The concentration of both nimbolide and iron oxide are conjugated in equal ratios for definite consistency.

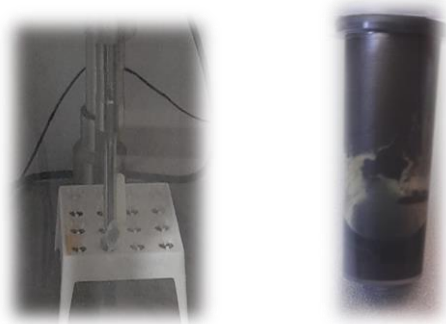


Fig.5.4: Nimbolide conjugated to the nanoparticle

5.5. UV CHARACTERIZATION OF THE CONJUGATED SAMPLE

The nanoparticle iron oxide was commercially obtained so the confirmation was already done to proceed with the conjugation. The conjugated sample was set to UV to see whether it had fused and was present along with the nimbolide sample or not.

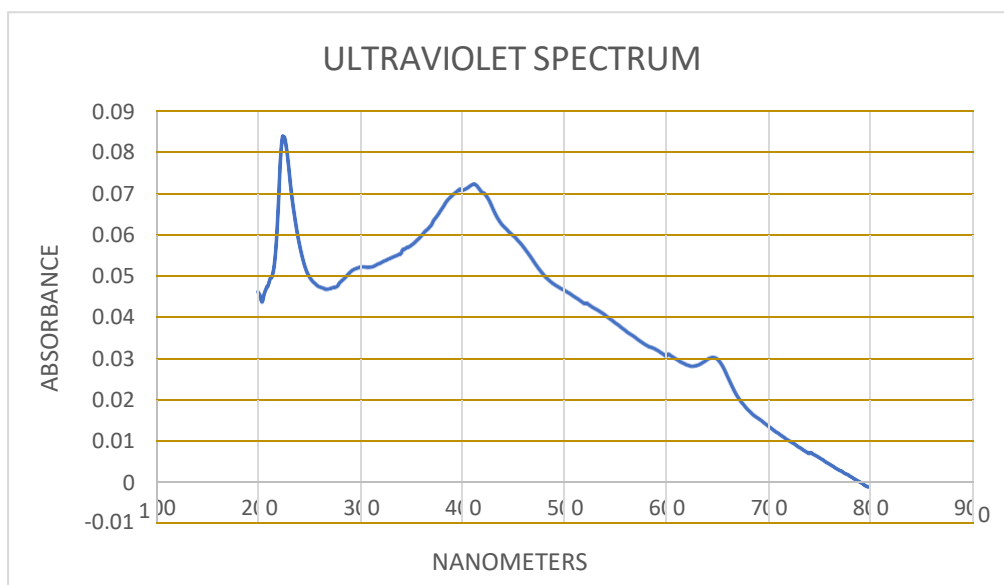


Fig.5.5. UV Characterization Of The Conjugated Sample

There are a few minor peaks as well along with nimbolide peaks indicating that nanoparticles are bound to the nimbolide sample.

The sample is then set ready for the antimicrobial testing process. The most preferred one is to do tests on gram-negative species as their cell permeability isn't easy compared to that of the gram-positive species. An environmental isolate of *E.coli* species is taken for the same. *E.coli* number 221 is chosen among the others.

5.6. FTIR ANALYSIS OF THE CONJUGATED SAMPLE

Fourier Transform Infrared Spectroscopy (FTIR) is a very crucial analytical technique used for characterizing nanoparticles conjugated with various molecules or functional groups.

FTIR is used to study the interactions between nanoparticles and conjugated molecules or ligands. Changes in the infrared spectra before and after conjugation can indicate the formation of new chemical bonds, hydrogen bonding, or other interactions between the nanoparticles and the attached molecules. Understanding these interactions is crucial for optimizing the design of nanoparticle-based systems for various applications, such as in drug delivery, sensing, and catalysis.

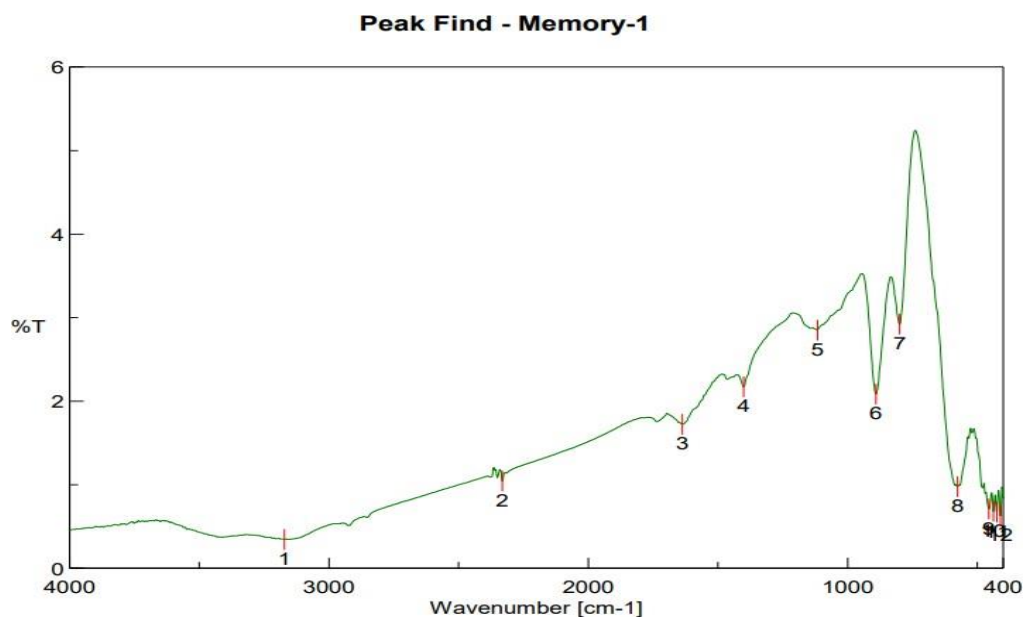


Fig.5.6: FTIR Analysis Report of conjugated sample

5.7.GRAM STAINING

Gram staining is a differential staining technique used in microbiology to classify bacteria into two broad categories: Gram-positive and Gram-negative. This technique was developed in 1884 by a Danish bacteriologist Hans Christian Gram which has been proved to be one of the most widely used staining techniques in microbiology laboratories.

The concept behind Gram staining lies in the fundamental structural differences between two major groups of bacteria: Gram-positive and Gram-negative bacterial species. The staining technique allows microbiologists to differentiate between these two groups based on the properties of their cell walls.

GRAM-POSITIVE BACTERIA:

These bacterial species with a thick layer of peptidoglycan in their cell walls tend to retain the crystal violet-iodine complex.

The peptidoglycan layer is surrounded by a cytoplasmic membrane.

Lack of an outer lipid membrane.

GRAM-NEGATIVE BACTERIA:

These bacterial species have a thinner layer of peptidoglycan in their cell walls and do not retain the primary stain, unlike Gram-positive bacteria.

The peptidoglycan layer is sandwiched between an inner cytoplasmic membrane and an outer lipid membrane.

The outer lipid membrane contains lipopolysaccharides, which contribute to the structural differences observed during staining.

5.7.1 Slide preparation:

Bacterial smear preparation: A thin, uniform bacterial smear is prepared on a glass slide from a culture grown on an agar medium.

Heat fixing the slides: The bacterial smear is air-dried and then heat-fixed by passing it through a flame or gently heating it. Heat fixation kills the bacteria and adheres them to the slide, preventing them from washing off during subsequent staining steps.

Staining Process: The slide is flooded with crystal violet, a purple-colored dye. Crystal violet binds to the peptidoglycan layer of the bacterial cell wall in all cells, staining them purple. After a short period, the excess stain is washed off with water.

- **Mordant (Iodine):** The slide is treated with an iodine solution (Gram's iodine). Iodine acts as a mordant, forming a complex with the crystal violet and enhancing its retention within the bacterial cells. This step is crucial for the differentiation of Gram-positive and Gram-negative bacteria.
- **Decolorization:** The slide is rinsed with alcohol or acetone to remove the stain from some bacteria while retaining it in others. This step is critical in distinguishing between Gram-positive and Gram-negative bacteria. Gram-

positive bacteria retain the crystal violet-iodine complex due to their thicker peptidoglycan layer, while Gram-negative bacteria lose the stain due to their thinner peptidoglycan layer and outer membrane. The decolorization step must be carefully timed to avoid over-decolorization, which can result in false-negative results.

- Counterstain (Safranin): The slide is then stained with a counterstain, usually safranin or fuchsin, which stains the decolorized Gram-negative bacteria pink or red. This step allows Gram-negative bacteria to be visualized.

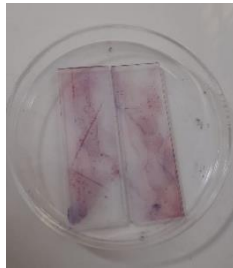


Fig.5.7: Prepared slide for staining

Examination: The stained slide is examined under a light microscope. Gram-positive bacteria appear purple, while Gram-negative bacteria appear pink or red.

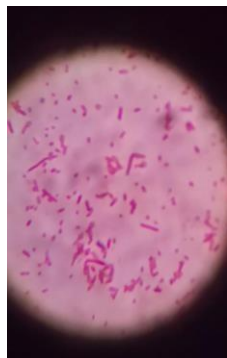


Fig.5.8: Visualization of the slide at 40X

5.8. DISC DIFFUSION METHOD

It is an antibiotic susceptibility test done in laboratories to usually check the antimicrobial properties of the specific drug or sample to be tested. The discs used are usually antibiotic, sterile discs, or filter paper discs. The preparation of agar plates for this particular process usually involves the usage of Mueller Hilton agar

(M.H. agar). This agar is chosen for the process because of its higher diffusion rates. It is said to permit better diffusion of antibiotics in the media as compared to other available media. Different concentrations of samples are loaded onto the discs and they are aseptically placed on agar surface using forceps. Any kind of mishandling can lead to contamination. Sterile discs were used for this process as the sample was an antibiotic. Different MICs were first used on discs starting from a lesser quantity of 0.2µl, 0.6µl, and 1µl of the pure nimbolide sample, nanoparticle sample, and nimbolide conjugated to nanoparticle samples

Medium	Concentration (g/ml)
L.B broth powder	3.9g
Distilled water	200ml

Table 5.1: L.B broth preparation

The L.B broth and distilled water were mixed in a flask and subjected to autoclaving for about 45mins to 1h. After autoclaving around 50 µl of the strain of *E.coli* was added to the flask and was kept in a shaker incubator at room temperature for about 24 to 48 h for the culture to grow properly.



Fig. 5.9: L.B broth inoculated with E.coli strain

Medium	Concentration
Beef infusion	300ml
Casein hydrolysate	1.6g
Starch	1.5g
Agar	1.5g
Distilled water	1000ml
PH level	7.3

Table 5.2: Mueller Hilton Agar

The disc diffusion method, also known as the Kirby-Bauer method, is a widely used technique in microbiology for determining the susceptibility of bacteria to antimicrobial agents. It involves the diffusion of antimicrobial agents from antibiotic-impregnated discs into a solid agar medium inoculated with a standardized bacterial culture. So in general it involves 6 major steps :

- 1) Preparation of agar plates using Mueller Hilton agar. 3.9g of agar was dissolved in a flask containing 100 ml of distilled water. It was cotton-plugged and autoclaved along with glassware for about 45 mins to 1h.
- 2) Once autoclaved, the agar was poured into the plates and left to solidify. After solidification, the bacterial strain of *E.Coli* was inoculated into the agar.
- 3) The sterile discs are soaked with a known concentration of the drug and are placed onto the agar.
- 4) Then the plates are incubated for about 24h at 37°C (room temperature).
- 5) Measurement of the zone of inhibition is done using a ruler and observations were noted accordingly.
- 6) Results were interpreted by comparing the diameters of the zone of inhibition for different discs soaked with nimbolide and nimbolide-nanoparticle conjugated samples.

CHAPTER 6: RESULT AND DISCUSSION

The Disc diffusion was carried out at different rates as mentioned above but the changes were very subtle enough to make any conclusion so, this process was repeated for the second time and the discs this time were soaked with much higher levels of nanoparticle conjugated nimbolide sample. The discs were soaked with around 10 μ l, 20 μ l, and 40 μ l of both samples. The higher MICs were promising enough to show correct results. So as expected the conjugated sample shows higher antimicrobial activities when a higher MIC sample is used, activity rate is quite lower when the quantities of MICs used are lesser than 10 μ l as per the observation.

6.1 DISC DIFFUSION RESULTS

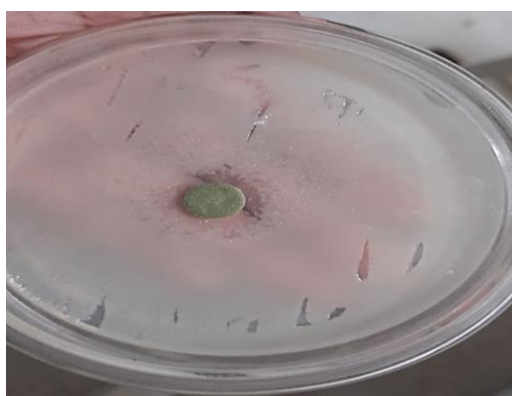


Fig.6.1: *Disc diffusion of pure nimbolide sample at highest MIC*



Fig 6.2: *Disc diffusion of pure nimbolide at lower MICs*

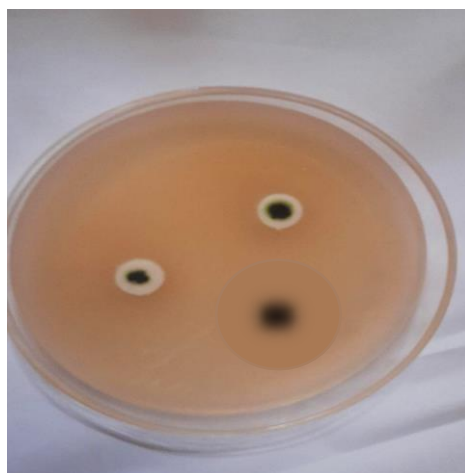


Fig.6.3: Disc diffusion of nanoparticle conjugated sample at higher MIC



Fig.6.4: Disc diffusion of nanoparticle conjugated sample at lower MICs

The interpreted result showed that the disc that is soaked entirely with a nimbolide-nanoparticle conjugated sample exhibited a maximum zone of inhibition.

40 μ l of the conjugated sample containing disc had the maximum effect and had a diameter of about 1.4cm or 14mm (Fig. 6.3). The second disc soaked with 20 μ l sample showed a zone of inhibition of about 0.8cm or 8mm. The third disc soaked with 10 μ l of the same sample showed the least 0.3 cm. Pure nimbolide-soaked discs of 10 μ l, 20 μ l, and 30 μ l showed very minimal levels of the zone of inhibition such as

0.2cm, 0.3cm, and 0.5cm respectively almost the least (Fig. 6.2) . When nimbolide-soaked discs with 0.2µl and 0.6µl were placed the zone of inhibition was almost none but when 40µl of pure nimbolide-soaked disc was placed it showed a zone of inhibition of about 1cm and seemed to work only at higher MICs (Fig. 6.1). These were visible results that were analyzed from the study.

MINIMUM INHIBITORY CONCENTRATION (MIC)	ZONE OF INHIBITION (DIAMETER)
Nimbolide At Lower MIC	0cm
0.2	
0.6	0cm
Nimbolide At Higher MIC	0.2cm
10	
20	0.3cm
30	0.5cm
Nimbolide At The Highest MIC	1cm
40	
Conjugated Sample - Low To High MICs	0.3cm
10	
20	0.8cm
40	1.4cm

Table 6.1: Zone of inhibition at different MICs

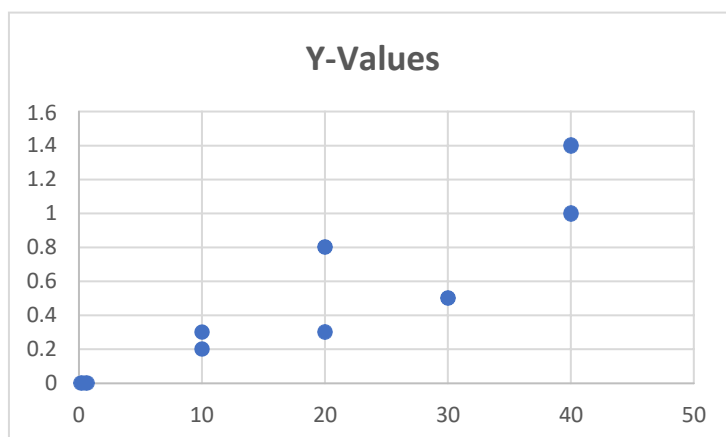


Fig. 6.5: MIC vs Zone of Inhibition-comparative Evaluation

6.2 FESEM ANALYSIS RESULTS

FESEM stands for Field Emission Scanning Electron Microscopy. It is a powerful technique commonly used in investigating the surface of solid materials at very high magnifications and resolutions. FESEM provides incredibly detailed images of the surface of materials at nanometer-scale resolution. This high resolution allows researchers to study the morphology, structure, and composition of materials with great precision.

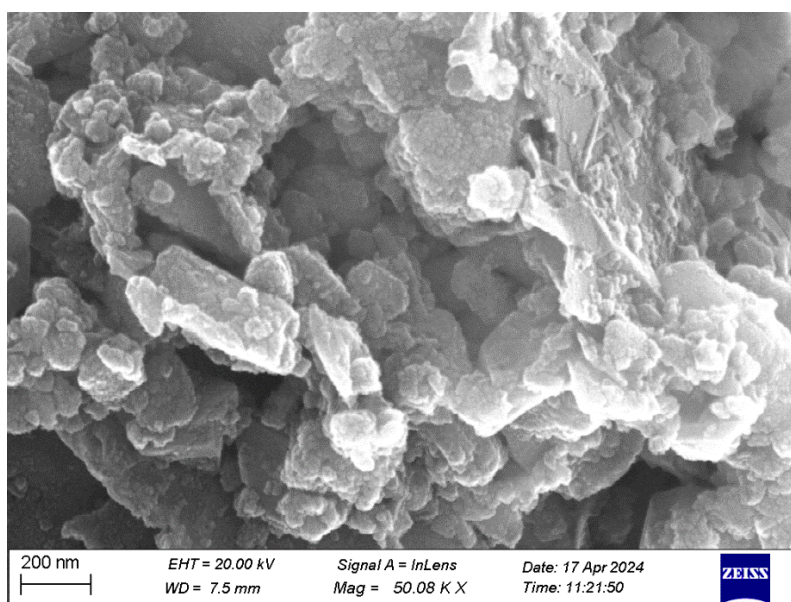


Fig. 6.5: FESEM Image of the conjugates sample

Interpretation:

It could be seen from the Field Emission Scanning Electron Microscopy (FESEM) images that the conjugated version of magnetic nanoparticles of Fe_3O_4 & nimbolide (the neem extracted compound) exhibited a asymmetrical surface topographical images at 200nm , 50.08 K X magnification .Such topographical morphology images of the composite illustrates the successful formation of the phyto- nanoparticle conjugates in the solution.

CHAPTER-7 SUMMARY AND CONCLUSION

We can conclude that the phytochemical that we have chosen for the objective of this project has shown good results in its antimicrobial activities. The main lead compound nimbolide has higher antimicrobial characteristics as per the biochemical testing but it is also noted that when the sample was conjugated along with a superparamagnetic iron oxide nanoparticle, its efficacy was comparatively higher than pure nimbolide sample. As we all know iron oxide, being a nanoparticle has a greater penetration capacity. It diffuses well with the nimbolide and aids in improved drug delivery systems. The objective of conjugating phytochemical with nanoparticle was to observe the rate of actions of the compound against the gram-negative bacteria. The disc soaked with 40 μ l of the conjugated sample shows much antimicrobial properties as compared to that of the pure nimbolide sample-soaked disc. The higher the amount of dosage higher the efficacy of action towards the pathogen. The nanoparticles as mentioned above can disrupt the cell membranes of the bacteria gram-negative or positive, they usually disrupt the cell membranes leading to the death of the cell eventually. The higher targeting specificity of nanoparticles makes it an even more ideal choice for any drug delivery system. Rather than affecting and acting on the surface of the cells they facilitate the cure from the root cause for its better application.

The FESEM imaging was done to critically analyze the binding and symmetry of the nanoparticle along with the phytochemical and it was found that they both were well bound together and a good symmetry was obtained indicating a better provision for drug delivery as the drug was well encapsulated within the nanoparticle that enhanced their efficacy and mode of action right at target sites.

The following are the key noted points of nanoparticles in drug delivery systems using these mechanisms:

Increased Penetration: Gram-negative bacteria have an outer membrane that acts as a barrier, making them less susceptible to many antibiotics. Nanoparticles can penetrate this outer membrane more effectively than conventional antibiotics alone, allowing them to deliver drugs directly to the bacterial cell.

Disruption of Cell Membrane: Some nanoparticles have inherent antimicrobial properties and can disrupt the integrity of the bacterial cell membrane. This disrupts

essential cellular functions and leads to bacterial death. Additionally, nanoparticles can facilitate the delivery of conventional antibiotics to the bacterial membrane, enhancing their efficacy.

Synergistic Effects: Nanoparticles can act synergistically with antibiotics, enhancing their antimicrobial activity against gram-negative bacteria. For example, silver nanoparticles have been shown to enhance the activity of antibiotics such as tetracycline and ampicillin against gram-negative bacteria.

Biofilm Disruption: Gram-negative bacteria form biofilms, which are dense colonies of bacteria encased in a protective matrix of extracellular polymeric substances. Biofilms usually prevent access to antibiotics and impair the host's immune functioning, making infections difficult to treat. Nanoparticles can disrupt biofilms and enhance the penetration of antibiotics into bacterial cells, making them more effective against biofilm-associated infections.

Targeted Delivery: Nanoparticles can be engineered to specifically target gram-negative bacteria, minimizing off-target effects and reducing the risk of antimicrobial resistance. Functionalization of nanoparticles with ligands that recognize specific bacterial surface receptors can enhance their specificity and efficacy.

REFERENCES:

- Arakha, M., Pal, S., Samantarrai, D., Panigrahi, T. K., Mallick, B. C., Pramanik, K., ... & Jha, S. (2015). Antimicrobial activity of iron oxide nanoparticle upon modulation of nanoparticle-bacteria interface. *Scientific reports*, 5(1), 14813.
- Arias, L. S., Pessan, J. P., Vieira, A. P. M., Lima, T. M. T. D., Delbem, A. C. B., & Monteiro, D. R. (2018). Iron oxide nanoparticles for biomedical applications: a perspective on synthesis, drugs, antimicrobial activity, and toxicity. *Antibiotics*, 7(2), 46.
- Armijo, L. M., Wawrzyniec, S. J., Kopciuch, M., Brandt, Y. I., Rivera, A. C., Withers, N. J., ... & Osiński, M. (2020). Antibacterial activity of iron oxide, iron nitride, and tobramycin conjugated nanoparticles against *Pseudomonas aeruginosa* biofilms. *Journal of Nanobiotechnology*, 18, 1-27.
- Bokel, M., Cramer, R., Gutzeit, H., Reeb, S. and Kraus, W., 1990. Tetranortriterpenoids related to nimbin and nimbolide from *Azadirachta indica* A. Juss (Meliaceae). *Tetrahedron*, 46(3), pp.775-782
- Cho, K., Wang, X. U., Nie, S., Chen, Z., & Shin, D. M. (2008). Therapeutic nanoparticles for drug delivery in cancer. *Clinical cancer research*, 14(5), 1310-1316.
- Couvreux, P. (2013). Nanoparticles in drug delivery: past, present and future. *Advanced drug delivery reviews*, 65(1), 21-23.
- D'Amico, C., Fontana, F., Cheng, R., & Santos, H. A. (2021). Development of vaccine formulations: past, present, and future. *Drug Delivery and Translational Research*, 11, 353-372.
- De Jong, W. H., & Borm, P. J. (2008). Drug delivery and nanoparticles: applications and hazards. *International journal of nanomedicine*, 3(2), 133-149.
- Dhanya, S. R., Kumar, S. N., Sankar, V., Raghu, K. G., Kumar, B. D., & Nair, M. S. (2015). Nimbolide from *Azadirachta indica* and its derivatives plus first-generation

cephalosporin antibiotics: a novel drug combination for wound-infecting pathogens. *RSC advances*, 5(109), 89503-89514.

Dobson, J. (2006). Magnetic nanoparticles for drug delivery. *Drug development research*, 67(1), 55-60.

El-Boubbou, K. (2018). Magnetic iron oxide nanoparticles as drug carriers: Preparation, conjugation and delivery. *Nanomedicine*, 13(8), 929-952.

Elumalai P, Ezhilarasan D, Raghunandhakumar S. Molecular targets of nimbolide for anti-cancer therapy: an updated review. *J Environ Pathol Toxicol Oncol* 2022; 41(2): 69–88

F Hasany, S., H Abdurahman, N., R Sunarti, A., & Jose, R. (2013). Magnetic iron oxide nanoparticles: chemical synthesis and applications review. *Current Nanoscience*, 9(5), 561-575.

Fazio, E., Santoro, M., Lentini, G., Franco, D., Guglielmino, S. P. P., & Neri, F. (2016). Iron oxide nanoparticles prepared by laser ablation: Synthesis, structural properties and antimicrobial activity. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 490, 98-103.

Gavas, S., Quazi, S., & Karpiński, T. M. (2021). Nanoparticles for cancer therapy: current progress and challenges. *Nanoscale research letters*, 16(1), 173

Guo, Y. G., Hu, J. S., & Wan, L. J. (2008). Nanostructured materials for electrochemical energy conversion and storage devices. *Advanced Materials*, 20(15), 2878-2887.

Heiligttag, F. J., & Niederberger, M. (2013). The fascinating world of nanoparticle research. *Materials today*, 16(7-8), 262-271.

Herrera-Calderon, O., Ejaz, K., Wajid, M., Shehzad, M., Tinco-Jayo, J. A., Enciso-Roca, E., ... & Chumpitaz-Cerrate, V. (2019). *Azadirachta indica*: Antibacterial activity of neem against different strains of bacteria and their active constituents as preventive in various diseases. *Pharmacognosy Journal*, 11(6s).

Hiremath, A., Murthy, A. A., Thipperudrappa, S., & KN, B. (2021). Nanoparticles filled polymer nanocomposites: A technological review. *Cogent engineering*, 8(1), 1991229.

Islas, J. F., Acosta, E., Zuca, G., Delgado-Gallegos, J. L., Moreno-Treviño, M. G., Escalante, B., & Moreno-Cuevas, J. E. (2020). An overview of Neem (*Azadirachta indica*) and its potential impact on health. *Journal of Functional Foods*, 74, 104171.

Kashif, M., Hwang, Y., Hong, G., & Kim, G. (2017). In vitro comparative cytotoxic effect of Nimbolide: A limonoid from *Azadirachta indica* (Neem tree) on cancer cell lines and normal cell lines through MTT assay. *Pakistan Journal of Pharmaceutical Sciences*, 30.

Kedar Kumar Rout, Sagar Kumar Mishra, Development of a Sensitive HPTLC Method for Quantification of Nimbolide in *Azadirachta indica* and Its Dosage Form, *Journal of Chromatographic Science*, Volume 52, Issue 9, October 2014, Pages 1089–1094

Latham, A. H., & Williams, M. E. (2008). Controlling transport and chemical functionality of magnetic nanoparticles. *Accounts of chemical research*, 41(3), 411-420.

Maragathavalli S, Brindha S, Kaviyarasi N, Annadurai B, Gangwar S. Antimicrobial activity in leaf extract of neem (*Azadirachta indica* Linn.). *Int J Sci Nat* 2012; 3(1): 110–3.

Mondal S, Bandyopadhyay S, K Ghosh M, Mukhopadhyay S, Roy S, Mandal C. Natural products: promising resources for cancer drug discovery. *Anti-Cancer Agents Med Chem (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)* 2012; 12(1): 49–75. doi: [10.2174/187152012798764697](https://doi.org/10.2174/187152012798764697)

Nagini, S., Nivetha, R., Palrasu, M., & Mishra, R. (2021). Nimbolide, a neem limonoid, is a promising candidate for the anticancer drug arsenal. *Journal of medicinal chemistry*, 64(7), 3560-3577.

Nasiruddin Ahmad Farooqui, Akalanka Dey, G N Singh, T S Easwari. Antibacterial potential of Nimbolide from *Azadirachta indica*. *Int J Pharm Pharm Sci* 2014;6 (5):636-8.

Navinraj, S., Boopathi, N. M., Balasubramani, V., Nakkeeran, S., Raghu, R., Gnanam, R., ... & Santhanakrishnan, V. P. (2023). Molecular Docking of Nimbolide Extracted from Leaves of *Azadirachta indica* with Protein Targets to Confirm the Antifungal, Antibacterial and Insecticidal Activity. *Indian Journal of Microbiology*, 63(4), 494-512.

Pal, S. (2014). *Antimicrobial activity of iron oxide nanoparticles* (Doctoral dissertation).

Parveen, S., Misra, R., & Sahoo, S. K. (2017). Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine in cancer*, 47-98.

Patra, N., Kar, D., Pal, A., & Behera, A. (2018). Antibacterial, anticancer, anti-diabetic and catalytic activity of bio-conjugated metal nanoparticles. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 9(3), 035001.

Rassu, G., Sorrenti, M., Catenacci, L., Pavan, B., Ferraro, L., Gavini, E., ... & Dalpiaz, A. (2023). Conjugation, prodrug, and co-administration strategies in support of nanotechnologies to improve the therapeutic efficacy of phytochemicals in the central nervous system. *Pharmaceutics*, 15(6), 1578.

Rosaline H, Kandaswamy D, Gogulnath D, Rubin M. Influence of various herbal irrigants as a final rinse on the adherence of *Enterococcus faecalis* by fluorescence confocal laser scanning microscope. *J Conserv Dent* 2013; 16(4): 352

Sarkar S, Singh RP, Bhattacharya G. Exploring the role of *Azadirachta indica* (neem) and its active compounds in the regulation of biological pathways: an update on molecular approach. *3 Biotech* 2021; 11(4): 1–12.

Shrestha, B., Tang, L., & Romero, G. (2019). Nanoparticles-mediated combination therapies for cancer treatment. *Advanced Therapeutics*, 2(11), 1900076.

Sivasankar, M., & Kumar, B. P. (2010). Role of nanoparticles in drug delivery system. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 1(2), 41-66.

Suttiarporn P, Choommongkol V. Microwave-Assisted Improved Extraction and Purification of Anticancer Nimbolide from *Azadirachta indica* (Neem) Leaves. *Molecules*. 2020 June 24;25(12):2913Doi:10.3390/molecules25122913. PMID: 32599926; PMCID: PMC7356096.

Wilczewska, A. Z., Niemirowicz, K., Markiewicz, K. H., & Car, H. (2012). Nanoparticles as drug delivery systems. *Pharmacological reports*, 64(5), 1020-1037.

Wylie, M. R., Windham, I. H., Blum, F. C., Wu, H., & Merrell, D. S. (2022). In vitro antibacterial activity of nimbolide against *Helicobacter pylori*. *Journal of ethnopharmacology*, 285, 114828.

Xu, Y., Baiu, D. C., Sherwood, J. A., McElreath, M. R., Qin, Y., Lackey, K. H., ... & Bao, Y. (2014). Linker-free conjugation and specific cell targeting of antibody functionalized iron-oxide nanoparticles. *Journal of materials chemistry B*, 2(37), 6198-6206.