

Microbial Resistant Coating of Infectious Corneal Lens Using Green-aided Nanoparticles Isolated from Plant Extracts

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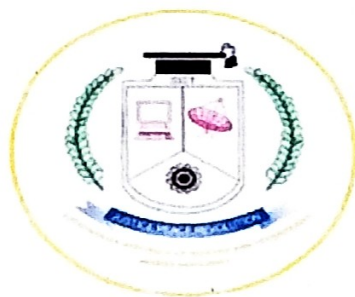
Bachelor of Pharmacy

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SCHOOL OF PHARMACY

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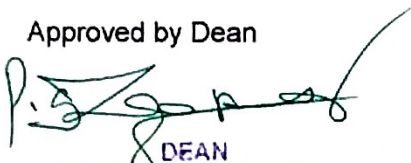
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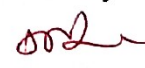


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Declaration

We do hereby declare that the work presented in the thesis entitled "**Microbial Resistant Coating of Infectious Corneal Lens Using Green-aided Nanoparticles Isolated from Plant Extracts**", submitted by us to the Sathyabama Institute of Science and Technology, Chennai-600119 for the award of the degree of Bachelor of Pharmacy(2019-2023) by Research is an original record of research work carried out by us under the supervision of our Internal guide **Dr. N. Delhiraj M. Pharm., Ph.D., Professor, School of Pharmacy** and our External guide **Dr. Sudha Uthaman M.Sc., Ph.D., Scientist "C", International Research Center**. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma, membership, fellowship, associateship etc.. In keeping with the general practice in reporting scientific observation, due acknowledgement has been made whenever the work described is based on the findings of other investigators.

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Abstract

Corneal lenses are considered as medical devices and they are used for both therapeutic and cosmetic purposes. The corneal lens usage is become an unavoidable one in day-to-day life. The people who are using corneal lenses are at greater risk of getting microbial keratitis due to unhygienic way of usage and maintenance. The regular usage of corneal lenses makes it dirt and developing risks due to pathogenic/microbial attack. Hence, it is mandatory to increase the life span of corneal lenses by using novel techniques. This present study aims at antibacterial wash of used infectious corneal lenses using silver and zinc nanoparticles derived from a plant extract of *Ocimum tenuiflorum* and *Clitorea termatea*, and *Aloe barbadensis miller* respectively. The used corneal lenses were arrived from microbial keratitis patients from Aravind Eye Hospital, Chennai. The newly derivated nanoparticles are used to wash the infected corneal lenses. The results showed that the green-aided antibacterial wash improves the antimicrobial efficacy of infected corneal lenses.

Key words

Ocimum tenuiflorum, *Clitorea termatea*, *Aloe barbadensis miller*, Microbial keratitis, Corneal lens, silver nanoparticles, Zinc Oxide nanoparticles, Antibacterial Activity.

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LIST OF ABBREVIATIONS

MK	Microbial Keratitis
CL	Corneal Lens
CLARE	Corneal Lens related Acute Red Eye
CLPU	Corneal Lens Peripheral Ulcers
NPs	Nanoparticles
AgNPs (or) Nano-Ag	Silver Nano Particles
ZnO NPs (or) Nano-ZnO	Zinc Oxide Nano Particles
HC1	Culture
HC2	Culture
AV	Aloe Vera
BP	Butterfly Pea
T	Tulsi
ROS	Reactive Oxygen Species
Rpm	Rotation Per Minute
°C	Degree Celsius
ml	Milliliters
μl	Microliters
G (or) g	Grams
H	Hours
HTS	High-throughput screening

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1. INTRODUCTION

1.1. Corneal Lenses

Corneal lenses are a convenient and comfortable alternative to eyeglasses for many people. Imperfect vision is one of the most common afflictions of the modern world.¹ Most corneal lens wearers adopt lenses for cosmetic reasons. But corneal lenses can also offer functional advantages over spectacles in many areas, such as in sports, work in humid environments, and in occupations in which spectacles are inconvenient or incompatible with required equipment, like special headgear. Since corneal lenses are introduced to the general public in the late 1950s, it has gained steadily while corneal lenses are usually a safe and effective form of vision correction, they are not entirely risk-free especially if they are not cared for properly. However, along with the recognized advantages of corneal lenses, there are some disadvantages of great potential significance.¹⁻² Corneal lens solutions made from distilled water and salt tablets or tap water and salt tablets were found to be the source of infections in 80 per cent of these patients.²



Fig.1.1. Corneal Lenses

Corneal lens materials and their physical properties have been investigated extensively over the years, with the promise of providing clear vision alongside comfortable and safe lens wear. Unfortunately, because of its constant exposure to the external environment, the ocular surface is susceptible to frequent microbial contamination.³ The "normal" bacteria on our skin, mouth, and nose usually do not cause any harm. But the combination of a lot of them on your corneal lenses and any small scratch on your eye (sometimes caused by sleeping in them) can be very dangerous.¹⁻²

About a third of people have *Staphylococcus aureus* in their nose. It is easily spread to your eyes by your hands through the corneal lenses, and it is stubborn and hard to treat. *Pseudomonas aeruginosa* is a bacterium that can cause a fast-moving severe infection of cornea.¹

However, adhesion and colonization of microorganisms, particularly bacteria, on corneal lenses continue to be the cause of conditions such as microbial keratitis (MK), corneal lens related acute red eye (CLARE) and corneal lens peripheral ulcers (CLPU).³

A recent study has demonstrated that *Pseudomonas aeruginosa* and *Staphylococcus epidermis* adhere much more strongly to unworn silicone hydrogel corneal lenses than to conventional hydrogel corneal lenses, and that adhesion of *Pseudomonas aeruginosa* is 20 times stronger than adhesion of *Staphylococcus epidermidis*. Developing antimicrobial coating for corneal lenses or corneal lens storage cases may decrease the risk of MK and other adverse events that are related to bacterial adhesion to corneal lenses. Antimicrobial coating for corneal lenses using the medicinal plants which have the antimicrobial property reduce the adhesion of the bacterial strains and appeared to be safe.³

1.1.1. Types of Corneal Lenses

Soft lenses are made of a plastic polymer and while they are prone to tearing, they are less likely to cause an infection of the eye. Soft lenses are available as disposable lenses where a new set of lenses are used on a daily or monthly basis. Used pairs can be discarded and the low cost of soft lenses makes it both an affordable and safe option.²

The hard corneal lenses, (also known as rigid gas permeable lenses), is made up of a silicone hydrogel and provide a better degree of correction for vision problems than soft lenses. Hard lenses are more durable than soft lenses and are necessary for presbyopia and some cases of astigmatism. Hard lenses can also be used for myopia and hypermetropia as well but there is a move towards daily wear soft lenses for these conditions.²

There are different problems that can arise as a result of wearing corneal lenses, some of which are mild and will resolve after discontinuing use. Other

complications can be severe causing permanent damage to the eye or leading to long term impairment of vision.²

1.1.2. Risk factors of Corneal Lenses

Many of the side effects associated with wearing corneal lenses tend to arise as a result of a few contributing factors. This includes: Wearing lenses overnight or for long periods of time without removing the lens or changing the lenses, Improper storage, handling, and use of lenses, Use of incorrect corneal lens accessories like the cleaning solution, Incorrect prescription leading to a poor fit., Poor quality composition materials in the manufacture of the lenses, Sharing of corneal lenses, Allergies, Dry eyes, Inflammation of the eyelids (blepharitis), Other medical conditions that can affect the eye, eyelid, or tear secretion (lacrimation), However, the factors above do not account for all the problems associated with corneal lens use.³

1.2. Microbial Keratitis

Bacterial keratitis or corneal ulcer is an infection of the corneal tissue caused by varied bacterial species. It can be an acute, chronic, or transient infectious process of the cornea with a variable predilection for topographical, anatomical, or geographical domains of the cornea. It can present as insidious progressive ulceration or rapidly deteriorating suppurative infection of any part of corneal tissue. The cornea can be invaded by various microorganisms like viruses, fungi, protozoa, and bacteria, but bacteria are most concerning due to rapidly progressive vision-threatening keratitis with irreversible visual sequelae.⁴



Fig. 1.2. Microbial Keratitis

When anatomical, mechanical, and antimicrobial barriers of microbial keratitis are breached, it invites vision-threatening keratitis. The past few decades have seen a rapid increase in corneal lens users globally, resulting in an increase in bacterial keratitis proportionately. The diagnosis rests on clinical and microbiological evaluation. With rapid advances in clinical diagnosis, growing research in molecular laboratory investigation, and targeted antimicrobial therapy, the visual morbidity has reduced to a decent extent, but it remains a significant cause of sight-threatening keratitis in underdeveloped and rural areas globally.⁴

1.2.1. Etiology of Keratitis

The main barriers of microbial infection are anatomical barriers in the form of the bony orbital rim, eyelids, intact conjunctival and corneal epithelium, mechanical barriers like tear film mucus layer and lacrimal system, and antimicrobial barriers like tear film constituents IgA, complement components, and lactoferrin, lysozymes and conjunctiva associated lymphoid tissue (CALT). When these barriers are disrupted, it may result in bacterial keratitis.⁵

Usage of corneal lenses is one of the major causes of bacterial keratitis. The various factors which cause bacterial keratitis are corneal lens overnight wear, overwear, inadequate cleaning of corneal lens, rinsing the corneal lens in tap water, contamination, lack of Corneal Lens hygiene, bandage corneal lenses, corneal Lens sharing, swimming with corneal lenses, Corneal Lens solution contamination and Corneal Lens induced trauma.⁶

The various extrinsic causes implicated are trauma, foreign body injury, chemical, mechanical, thermal injuries, insect fall, previous ocular, eyelid surgery, immunosuppression, drug-induced with corticosteroids and NSAIDs and substance abuse.⁷

Varied ocular surface diseases, either local or systemic, can also result in bacterial keratitis. The various local factors are dry eyes, corneal suture-related infection, abnormalities of eyelid anatomy and function, trichiasis, blepharitis, chronic dacryocystitis, ectropion, entropion conjunctivitis, lagophthalmos neurotropic keratopathy, recurrent corneal erosions, epithelial defect, secondary bacterial keratitis after viral keratitis, bullous keratopathy.⁷

The systemic conditions predisposing to bacterial keratitis are diabetes mellitus, malnourishment, connective tissue or autoimmune pathologies, Steven-Johnson syndrome (SJS), ocular mucous membrane pemphigoid (OMMP), atopic dermatitis, xerophthalmia, blepharoconjunctivitis, 5th and 7th cranial nerve palsy, graft versus host disease, immunosuppression (AIDS) and chronic alcoholism.⁷

1.3. Medicinal plants

Plants have been used for treatment various diseases since long time. Biological active compounds are obtained by extracting mainly the leaves, the fruits, the stems, or roots of medicinal plants. Recently, WHO (World Health Organization) estimated that 80 percent of people worldwide rely on herbal medicines for some aspect of their primary health care needs. According to WHO, around 21,000 plant species have the potential for being used as medicinal plants.⁸

To increase the application of plant extracts, a technology must be performed in formulation development of plant extracts. Plant extract loaded NPs offers numerous advantages because of their size and unique physicochemical characteristics. Moreover, plant extract loaded NPs can be used to decrease plant extracts toxicity, to provide targeted drug delivery and to solve stability related problems.⁸

1.4. Nanoparticles

Nanoparticle deals with the branch of the science of manipulating matter on an atomic or molecular scale. In recent years, nanotechnology has shown a rapid and successful growth, since it allows known materials to be developed with different properties. Decreasing the size of any material to nanoscale may change its intrinsic properties. Thus, the properties of a nanostructured material can be quite dissimilar from those of the bulk material, making it suitable for different applications. In particular, metallic nanoparticles (NPs) such as Silver and Zinc NPs (AgNPs and ZnNPs) have been applied in a wide variety of fields, including medicine, agriculture, bioengineering, among others, because they have been proven and recognized as antibacterial and biocide agents.⁹

At present, the nanomaterial field has generated NPs using green synthesis. Green synthesis of nanoparticles represents an advance over other methods because it is simple, cost-effective, and relatively reproducible, and often results in more stable materials. Hence, nowadays, many researchers are diverting themselves from using synthetic methods. an alternative option for synthesizing nanoparticles is by applying plants and their parts. Hence due to these advantages, green synthesis is gaining extreme importance in all the fields focusing on a greener environment.⁹

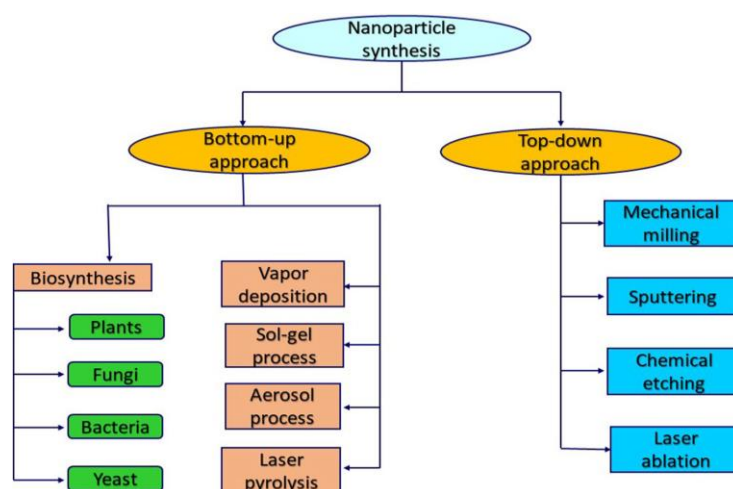


Fig. 1.3. Nanoparticles synthesis

Green synthesis of nanoparticles has many potential applications in environmental and biomedical fields. Green synthesis aims in particular at decreasing the usage of toxic chemicals. For instance, the use of biological materials such as plants is usually safe. Plants also contain reducing and capping agents.⁹

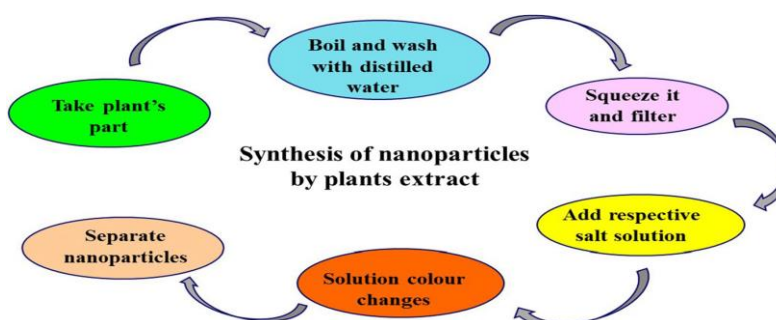


Fig. 1.4. green synthesis of nanoparticles using plants extract

The three foremost conditions for the synthesis of nanoparticles are the selection of green or environment-friendly solvent, a good reducing agent, and a harmless material for stabilization. For the synthesis of nanoparticles, extensive synthetic routes have been applied in which physical, chemical, and biosynthetic routes are very common. Generally, the chemical methods used are too expensive and incorporate the uses of hazardous and toxic chemicals answerable for various risks to the environment. The biosynthetic route is a safe, biocompatible, environment-friendly green approach to synthesize nanoparticles using plants and microorganisms for biomedical applications.⁹

1.5. Isolation and Identification of unknown isolates

1.5.1. Isolation of isolates taken from infected lenses

The process of obtaining a pure culture by separating one species of microbe from a mixture of other species, is known as isolation of the organisms

1.5.1.1. Methods of isolation

1. Streaking
2. Plating
3. Dilution
4. Enriched procedure, and
5. Single cell technique

1.5.2. Identification of isolates taken from infected lenses

1.5.2.1. Gram Staining

The differences in Gram-positive and Gram-negative bacteria cell wall composition account for the Gram staining differences. Gram-positive cell wall contains a thick layer of peptidoglycan with numerous teichoic acid cross-linking, which resists decolorization. In aqueous solutions, crystal violet dissociates into CV⁺ and Cl⁻ ions that penetrate through Gram-positive and Gram-negative cell walls. The CV⁺ interacts with negatively charged components of bacterial cells, staining the cells purple. When added, iodine (I⁻ or I₃⁻) interacts with CV⁺ to form large crystal violet-iodine (CV-I) complexes within the cytoplasm and outer layers

of the cell. The decolorizing agent (ethanol or an ethanol and acetone solution) interacts with the lipids of both gram-positive and gram-negative bacteria membranes. The outer membrane of the Gram-negative cell is lost from the cell, leaving the thin peptidoglycan layer exposed. With ethanol treatment, gram-negative cell walls become leaky and allow the large CV-I complexes to be washed from the cell. The highly cross-linked and multi-layered peptidoglycan of the gram-positive cell is dehydrated by the addition of ethanol. Thus, ethanol treatment traps the large CV-I complexes within the cell. After decolorization, the gram-positive cell remains purple. In contrast, the gram-negative cell loses the purple color and is only revealed when the counterstain, the positively charged dye safranin, is added.¹²

1.5.2.1.1. Steps of Gram Staining

- 1) **Fixation of clinical materials** to the surface of the microscope slide either by heating or by using methanol. (Methanol fixation is recommended rather than heat fixation. Methanol fixation preserves the morphology of host cells and bacteria. Heating the slide causes cell distortion, could increase cell debris, and may cause erroneous Gram stain results.).
- 2) **Application of the primary stain (crystal violet)**. Crystal violet is a dark blue to purple dye. It stains all cells blue/purple.
- 3) **Application of mordant**: The iodine solution (mordant) is added to form a crystal violet-iodine (CV-I) complex; all cells continue to appear blue.
- 4) **Decolorization step**: The decolorization step distinguishes gram-positive from gram-negative cells. The organic solvent such as acetone or ethanol extracts the blue dye complex from the lipid-rich, thin-walled gram-negative bacteria to a greater degree than from the lipid-poor, thick-walled, gram-positive bacteria. The gram-negative bacteria appear colorless, and gram-positive bacteria remain blue.
- 5) **Application of counterstain (safranin)**: The red dye safranin stains the decolorized gram-negative cells red/pink; the gram-positive bacteria remain blue.¹²

1.5.2.2. Catalase test

This test is performed to determine the ability of microorganisms to produce catalase that degrades hydrogen peroxide. During respiration, many microorganisms produce hydrogen peroxide and other reactive oxygen intermediates like superoxide and halite ions. Accumulation of these is toxic unless inactivated enzymatically. The enzyme catalase inactivates highly toxic H_2O_2 to water and oxygen.¹²

1.5.2.3. Citrate utilization test

This test is performed to determine the strain's ability to use citrate as an exclusive source of carbon and ammonia as its only source of nitrogen. Test strain is cultured in Simmon's citrate medium that is a modified form of Simmon's citrate containing sodium citrate, an ammonium salt, bromothymol blue an indicator, and agar.¹²

1.5.2.4. Mannitol Motility test

Motility by bacterium is mostly demonstrated in a semi solid agar medium. In semi-solid agar media, motile bacteria 'swarm' and give a diffuse spreading growth that is easily recognized by the naked eye. The medium mainly used for this purpose is SIM medium (Sulphide Indole Motility medium). The inoculum is stabbed into the center of a semisolid agar deep. Motile bacteria grow throughout the entire medium, whereas others show small areas or nodules that grow out from the line of inoculation. The non-motile bacteria will only grow in the soft agar tube and only the area where they are inoculated.¹²

1.5.2.5. Starch Hydrolysis Test

Starch molecules are too large to enter the bacterial cell, so only bacteria that secrete exoenzymes (α -amylase and oligo-1,6-glucosidase) are able to hydrolyze starch into subunits (dextrin, maltose, or glucose). These molecules are readily transported into the bacterial cell to be used in metabolism. When bacteria capable of producing α -amylase and oligo-1,6-glucosidase are grown on starch agar, they secrete enzymes into the surrounding areas and hydrolyze the starch.

As no amylose is present in the medium surrounding the bacterial colony, clearing around the bacterial growth is seen.¹²

1.5.2.6. Carbohydrate Tests

When microorganisms ferment carbohydrate, an acid or acid with gas are produced. Depending upon the organisms involved and the substrate being fermented, the end products may vary. The production of the acid lowers the pH of the test medium, which is detected by the color change of the pH indicator. Color change only occurs when a sufficient amount of acid is produced, as bacteria may utilize the peptone producing alkaline by-products.¹²

1.6. Characterization by UV-Visible Spectroscopy

UV-visible spectroscopy used to confirm the formation of various types of nanoparticles by measuring Plasmon resonance and evaluating the collective oscillations of conduction band electrons in response to electromagnetic waves. It provides information about the size, structure, stability, aggregation of the nanoparticles. Metal nanoparticles are associated with specific absorbance bands in characteristic spectra when the incident light enters into resonance with conduction band electrons on the surface of the nanoparticle.¹²

1.7. Enumeration of viable bacterial count

Bacteria enumeration is the process of determining the number of bacterial cells in a given sample. The counting of bacterial cells has four categories based on the purpose of the experiment: direct, indirect, viable, and total cell count. Methods have been developed to count the numbers of bacteria are standard plate count, turbidimetric method, and direct microscopic count.

1.7.1. BacTiter-Glo™ Microbial Cell Viability Assay: It is a homogeneous method to determine the number of viable bacterial cells in culture based on quantitation of ATP. The assay is designed for either single-tube or multiwell-plate formats for high-throughput screening (HTS).

The assay procedure involves adding BacTiter-Glo™ Reagent directly to bacterial cells in medium and measuring luminescence. Washing cells, removing culture medium and performing multiple pipetting steps are not required.¹²

2. LITERATURE REVIEW

- Linyan Nie et al¹³ revealed that the development of antibiotic resistance, novel approaches have been developed to combat corneal keratitis. Corneal lenses were initially developed to correct vision. Although silicon hydrogel-based corneal lenses protect the cornea from hypoxic stress from overnight wear, wearing corneal lenses was reported as an essential cause of corneal keratitis.
- Stapleton F et al¹⁴ reviewed that Individual differences in susceptibility to microbial keratitis may be partly explained by differences in single-nucleotide polymorphisms in certain cytokine genes, particularly those with a proven protective role in corneal infection.
- Bharat Gurnani et al²⁴ investigated that Bacterial keratitis (corneal ulcer) is a sight-threatening corneal lens complication. Either untreated or severe bacterial keratitis may result in perforation and endophthalmitis.
- Philip Maier et al¹⁵ has investigated that Corneal infiltration that is diagnosed early is often self-limited, but advanced bacterial infection usually requires intense topical antibiotic treatment. Some severe infections can only be eradicated by emergency corneal transplantation; this is the case in 20–30% of fungal and bacterial infections.
- Ahuja M et al¹⁶ reviewed that Microbial keratitis is a rare but most significant complication is discussed in this article. *Pseudomonas aeruginosa* and staphylococci are the most common organisms cause infective keratitis. Fungi and antibacterial are also responsible. Ocular pain, conjunctival infection. Antibacterial can be cultured from corneal scrapes. Immunologically based fluorescein labelling techniques appear to be more sensitive than simple staining. The treatment consists of medical and surgical intervention.
- Al-Mujaini A et al¹⁷ highlighted that the epidemiological data reveals the universal occurrence of this disease. With advances in the understanding of its pathogenesis, laboratory investigations like immunohistochemistry, fluorescent

microscopy, enzyme immunoassays and molecular biology, and the availability of fourth generation antibiotics, the overall visual outcome in bacterial keratitis has improved with time. Particular attention should be given to this condition as it can progress very rapidly with complete corneal destruction occurring within 24–48 hours.

- Fleiszig SMJ et al¹⁸ highlighted the perspective on the complex pathogenesis of microbial keratitis, the factors that have prevented a better understanding of this disease, and new approaches being used to tack this important clinical problem.
- Daniell M et al¹⁹ investigated that predisposing factors, clinical and microbiological characteristics of bacterial keratitis in current practice.
- David J. Evans et al²⁰ highlighted that Microbial keratitis is an acute, and potentially serious complication of corneal lens wear. Increasing resistance of infecting bacteria to antimicrobial therapy complicates the treatment of microbial keratitis. Even with successful therapy, visual outcomes of microbial keratitis often involve reduced visual acuity.
- Mohammadpour M et al²¹ highlighted that Microbial keratitis is a rare and acute corneal disease, a potentially blinding condition for those who do not receive rapid and appropriate treatment. It has been shown that the use of corneal lenses is recognized as one of the major risk factors for developing this condition in western and developing countries.
- Lim CH et al²² reviewed that independent risk factors for corneal lens-related microbial keratitis include poor hand hygiene, occasional overnight wear, and type of lens care solution. Prolonged overnight or extended corneal lens use was infrequent in this population.

3. AIM AND OBJECTIVE

Aim of the work

This work aims at improving the life span of corneal lenses which are used in a day-to-day life by the keratitis patients and when it is used for cosmetic purposes. Though nano-coated lenses are available in the market, those are costly and chemically-treated that are not prescribed for a healthy way of using it. Hence, in our study, it was proposed to coat the corneal lenses with nanoparticles which are synthesized from a natural way called green synthesis. The objectives of this work are listed below.

Objectives of the work

Objective-1: To suitably identify the plants that are rich in nanoparticles with more capability to fight against microbes/pathogens.

Objective-2: To take out the plant extract and using which synthesis of nanoparticles by green synthesis.

Objective-3: To coat the nanoparticles using nanoparticles that are synthesized from plant extract.

Objective-4: To examine and characterize the anti-microbial efficacy of cost-effective nanoparticles synthesized from plant extracts.

4. EXPERIMENTAL WORK

4.1. Plant Profile of *Ocimum tenuiflorum*

Ocimum tenuiflorum (also known as *Ocimum sanctum* L, Tulsi) has been used as medicine for more than thousands of years in Indian traditional medicine, Ayurveda for its diverse healing properties. *Ocimum tenuiflorum* is a member of Lamiaceae family consists of almost 200 species of herbs and shrubs and is graded high among some of the astonishing herbs for having tremendous medicinal potentialities. There are large numbers of distinct species and varieties falls in this genus.²³



Fig.4.1. *Ocimum tenuiflorum*

Scientific classification:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Lamiales

Family: Lamiaceae

Genus: *Ocimum*

Species: *Ocimum tenuiflorum*

Constituents: linalool, alkaloids, ursolic acid, glycosides, carvacrol, tannins, rosmarinic acid, and aromatic compound.

Pharmacological Activity: Antibacterial Activity, Antidepressant Activity, Antidiabetic Activity, Anticancer Activity, Antifungal, Anthelmintic Activity, Antioxidant Activity.²⁴

4.2. *Clitoria ternatea*

It is commonly known as butterfly pea, is a perennial herbaceous plant from the Fabaceae family. It has recently attracted a lot of interest as it has potential applications both in modern medicine and agriculture, and as a source of natural food colorants and antioxidants. It has long been cultivated as a forage and fodder crop, and early studies assessed the plant for these purposes.²⁸



Fig. 4.2. *Clitoria ternatea*

Scientific classification:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Tracheophyta

Class: Equisetopsida C. Agardh

Order: Fabales

Family: Fabaceae

Genus: *Clitoria*

Species: *Clitoria ternatea* L.

Common Name:

Tamil- Sankupushpam

Telugu- Gentana

Malayalam- Sangu Pushpam

Marathi- Gokarna

Hindi- Aparajitha

Constituents: Tannins, phlobatannin, carbohydrates, saponins, triterpenoids, phenols, flavanoids, flavonol glycosides, proteins, alkaloids, anthraquinone, anthocyanins, cardiac glycosides, Stigmast-4-ene-3,6-dione, volatile oils and steroids.

Pharmacological Activity: Antimicrobial Activity, Antiparasitic Activity, Anti-inflammatory Activity, antipyretic Activity, analgesic Activity, Anticancer Activity.²⁸

4.3. *Aloe Barbadensis miller*

Aloe vera (*Aloe barbadensis* Miller) a perennial succulent belonging to the Liliaceal family, is a cactus-like plant that grows in hot, dry climates. Aloe gel is the mucilaginous jelly obtained from parenchyma cells of the Aloe vera plant. For many years, Aloe vera has been reported to possess anti-inflammatory, UV protective, antibacterial, and wound healing properties. Aloe vera has been used for medicinal purposes in several cultures for millennia and it contains 75 potentially active constituents: vitamins, enzymes, minerals, sugars, salicylic acids, lignins, saponins, amino acids and has been used for medicinal purposes in several cultures for millennia.³⁰



Fig.4.3. *Aloe Barbadensis miller*

Scientific classification:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Class: Monocotyledonae

Order: Liliales

Family: Aloaceae

Genus: Aloe

Species: Aloe vera

Common Name:

Tamil- Kathalai

Telugu- Kalabanda

Kannada-Lolisara

Marathi- Koarphada

Hindi- Gwarpatha

Constituents: Fibres, proteins, organic acids, minerals, monosaccharides, and polysaccharides

Pharmacological Activity: Antimicrobial Activity, anti-inflammatory and anti-arthritis activity and hypoglycaemic effects, Anticancer Activity.³⁰

4.4. Materials

A pair of infected corneal lenses was brought from the Aravind Eye Hospital, Chennai. And the infected bacteria were cultured by streak method and grown in the nutrient broth.

The plant *Ocimum tenuiflorum*, *Clitoria ternatea*, *Aloe Barbadensis* miller were collected from Madhavaram Botanical Garden, Chennai, Tamil Nadu.

4.4.1. Chemicals

Table.4.1. Chemicals used

Acetone	SRL Pvt. Ltd.
Silver nitrate	Labchem Pvt. Ltd.
Biofilm	Labchem Pvt. Ltd.
Nutrient Agar	HiMedia Laboratories Pvt. Ltd.
Nutrient Broth	HiMedia Laboratories Pvt. Ltd.
Hydrogen Peroxide	SRL Pvt. Ltd.
Galactose Disc	Labchem Pvt. Ltd.
Lactose Disc	Labchem Pvt. Ltd.
Dextrose Disc	Labchem Pvt. Ltd.
Simmon Citrate Agar	HiMedia Laboratories Pvt. Ltd.
Starch Agar	HiMedia Laboratories Pvt. Ltd.
Ethanol	SRL Pvt. Ltd.
Gram's Iodine	Labchem Pvt. Ltd.
Zinc oxide	Labchem Pvt. Ltd.
Phenol Red	SRL Pvt. Ltd.
Acridine Orange	SRL Pvt. Ltd.
Agar powder	HiMedia Laboratories Pvt. Ltd.

4.4.2. Instruments

- ❖ Nikon 500 Epifluorescence Microscope
- ❖ Jasco V-670 UV-Visible Spectrometer

4.5. Methods

4.5.1. Preparation of Plant extracts

4.5.1.1. Preparation of *Ocimum tenuiflorum* leaf extract

250g Fresh leaves of *Ocimum tenuiflorum* were taken with 100 ml of acetone and kept aside at room temperature (25°C) for seven days in the dark. Then, the leaves were crushed & filtered through Whatman No.1 filter paper. The filtrate was sonicated and centrifuged at the rate of 10,000 rpm for ten minutes. The supernatant was an acetone extract.²⁴



Fig.4.4. fresh leaves of *Ocimum tenuiflorum*



Fig. 4.5. dry powdered leaves of *Ocimum tenuiflorum*

4.5.1.2. preparation of *Clitoria ternatea* extract (maceration method)

- i. 20g of fresh butterfly pea flowers are taken and chopped in to pieces.
- ii. Chopped flowers in to a beaker and add 100ml of distill water in to it
- iii. (Soak) Macerate the flowers up to 1 hour.
- iv. Filter the plant extract using Whatman filter paper
- v. Transfer it to a conical flask.²⁹



Fig. 4.6. fresh flowers of *Clitoria ternatea*



Fig. 4.7. dried flowers of *Clitoria ternatea*

4.5.1.3. Preparation of *Aloe barbadensis miller* leaf extract

20g of aloe vera leaves were cut into small pieces and it is dissolved in 100ml of deionized water. This extract was boiled at around 60°C for 10 minutes until the water turns green. Then the extract is filtered and used as reducing agent and capping agent for nanoparticle synthesis.³²



Fig.4.8. Skin removed aloe vera gel

4.5.2. Synthesis of Nanoparticles

4.5.2.1. Synthesis of Silver Nanoparticles using *Ocimum tenuiflorum* leaf extract

Silver nanoparticles are prepared by adding 10 mL of the acetone leaf extract of *Ocimum tenuiflorum* (20 g/100ml) to 40 mL of acetone silver nitrate solution (1 mM) in a 100 ml beaker. For this, 40 ml acetone solution of silver nitrate is taken in 100 ml glass beaker and 10 ml of extract is added dropwise at 50°C, under continues stirring conditions for 10 min using magnetic stirrer. The change in colour from colourless to dark brown gives the primary indication about the formation of silver nanoparticles. Afterwards, the suspension of Ag nanoparticles is centrifuged at 8000 rpm for 20 min and impurities are castoff without disturbing the silver nanoparticles.²⁴

4.5.2.2. Synthesis of Silver Nanoparticles using *Clitoria ternatea* flower extract

To achieve green synthesis of AgNPs, aqueous extract of *C. ternatea* was used as a reducing and stabilizing agent. The AgNPs were prepared by dropwise addition of 4.00 mL of 3.0 mM AgNO₃ to 20 mL of the extract. The solution was adjusted to pH 10 using 0.1 M NaOH and then heated at 50°C for 15 minutes. The reduction of Ag⁺ to Ag⁰ was confirmed by the colour change of the silver nitrate solution from colourless to deep brown. After the synthesis of AgNPs, the solution containing the

nanoparticles was centrifuged at 15,000 rpm for 15 minutes to separate the AgNPs from the solution. The supernatant was recentrifuged and the AgNPs was washed using DI water. The dried AgNPs were kept in a microtube for further study.²⁹

4.5.2.3. Synthesis of Zinc Nanoparticles using *Aloe barbadensis miller* leaf extract

6g of Zinc sulphate was dissolved in 150 ml of deionized water. 20ml of leaf extract was added to the 150 ml of Zinc sulphate solution and stirred for 3 hours at 60°C. Finally, an off-white opaque solution was obtained. This solution was centrifuged at 5000 rpm for 10 minutes and dried it to obtain a powder of Zinc oxide nano particle.³¹

4.6. Bacteriological Screening

Infected corneal lenses which are used by Microbial Keratitis patients were arrived from Aravind Eye Hospital, Chennai. The biofilm present on the surface of the corneal lenses was scrapped using a sterile brush into a buffer solution. The biofilm was inoculated and the predominant single colonies were isolated for further analysis

4.6.1. Gram staining

4.6.2. Catalase test

In a set of test tubes, nutrient agar was taken as growth medium and sterilized by autoclaving at 15 lb. pressure for 15 minutes. The nutrient agar slants were inoculated with isolated culture. A nutrient agar slant without inoculation was kept as control. All the slants were incubated at 37°C for 24h and 3-4 drops of hydrogen peroxide was added on the growth of each slant culture. The culture was observed for the appearance or absence of gas bubbles.¹²

4.6.3. Citrate utilization test

Simmon's citrate agar media was sterilized and the slants were prepared. The slants were inoculated with isolated culture to be identified. The uninoculated tubes were kept as control. The tubes were incubated at 37°C for 24-48h. Slant culture was observed for the change in colour from green to blue.¹²

4.6.4. Mannitol Motility test

Mannitol motility test Medium is designed to differentiate bacteria on the basis of their mobility and ability to ferment mannitol. Motile bacteria produce diffused growth throughout the medium while non-motile bacteria grow only along the line of inoculation. Fermentation of mannitol produces acidity in the colour change from red to yellow.¹²

4.6.5. Starch Hydrolysis Test

Starch agar media was prepared and sterilized using autoclave at 15 lbs. for 15 min. The media was poured into Petri plates and allowed to solidify. The test organism was inoculated on to the plate with a sterile transfer loop. The plate was incubated at 37°C for 48h. After incubation the plate was flooded with Gram's iodine. Plate was observed for clear zone around the test organism.¹²

4.6.6. Carbohydrate Tests

Fermentation medium (trypticase, sodium chloride, phenol red and a carbohydrate) was prepared with specific carbohydrates such as glucose, dextrose, maltose, lactose, galactose and sucrose and the media were sterilized. Each of specified fermentation tubes of media was labeled and inoculated with isolated cultures to be identified and one uninoculated tube with each fermentation broth was kept as comparative control. All the tubes were incubated at 37°C for 24-48h and observed for color change due to production of acid.¹²

4.7. Fluorescent microscopic images of corneal lenses coated with nanoparticles

The newly purchased corneal lenses in duplicate were coated with Ag and Zn nanoparticles derived from plant extracts were exposed to pathogenic culture which was scraped from the infected corneal lenses. The lenses were incubated at 37°C for 24 hours. Then the corneal lenses were stained with 0.1% Acridine Orange and left dry for 15 minutes. Acridine Orange is a nucleotide fluorescent dye which intercalates with DNA, and emits orange fluorescence. The air-dried lenses were washed with water and examined under epifluorescence microscope.¹²

5. RESULTS AND DISCUSSION

5.1. Enumeration of Viable Bacterial Count

Fresh corneal lenses were exposed to the bacterial cultures (Hospital cultures) obtained from infected corneal lenses for 24 hours and the culture was examined for viable bacterial count at Indira Gandhi Centre for Atomic Research, Kalpakkam. The test was performed in triplicate and the results are shown in Table 5.1.

Table 5.1. Total Viable Count

Specimen identification	Culture - Nanoparticles (counts)	Culture + Nano-Ag from <i>Ocimum tenuiflorum</i> (counts)	Culture + Nano-Ag from <i>Clitoria ternatea</i> (counts)	Culture + Nano-Zn from <i>Aloe barbadensis miller</i> (counts)
Hospital Culture (HC1)	1.13×10^6	1.32×10^4	7.34×10^3	6.64×10^4
Hospital Culture (HC2)	3.52×10^7	3.76×10^3	4.57×10^3	3.76×10^4

The number of viable bacteria was more on control culture when compared to nanoparticles admixed culture in all the three cases. The Nano-Ag extracted from Butterfly pea flower was more efficient compared to Nano-Ag extracted from Tulsi leaves. Compared to control culture, 2 orders, 3 orders and 4 orders decrease in viable count was observed for Aloe, Butterfly pea flower and Tulsi respectively.

5.1.2. Isolation and Identification of unknown isolates taken from infected lenses

The most predominant bacterial colonies were identified and it was isolated. The isolated bacterial colonies were grown in a petriplate by streaking method and then stored for further biochemical analysis. Based on the results observed through the identification methods the results are drawn. The figures of different biochemical procedures are shown in Figures 5.1 to 5.6 the results are shown in Table 5.2.

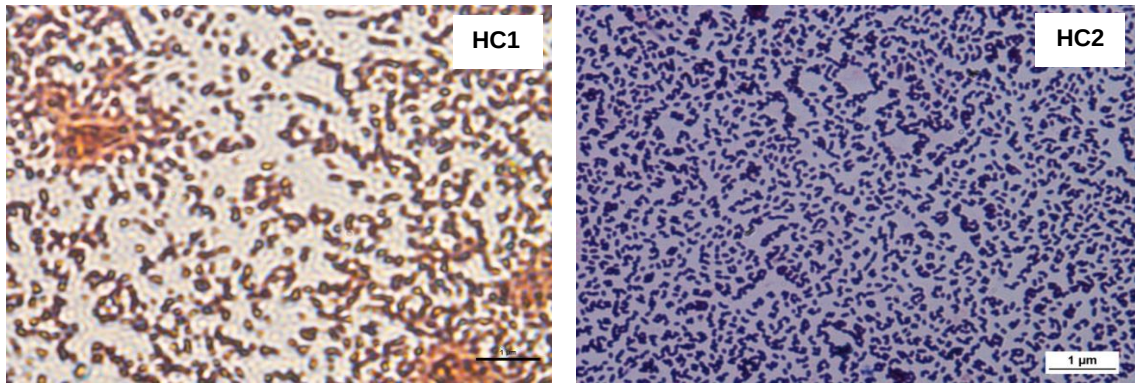


Fig. 5.1. Gram staining images

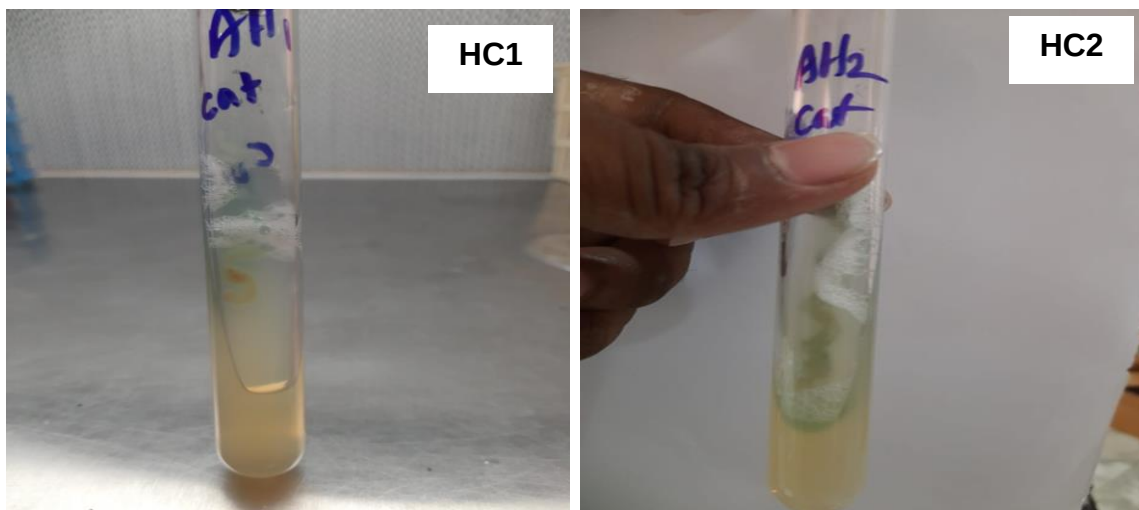


Fig. 5.2. Catalase Test

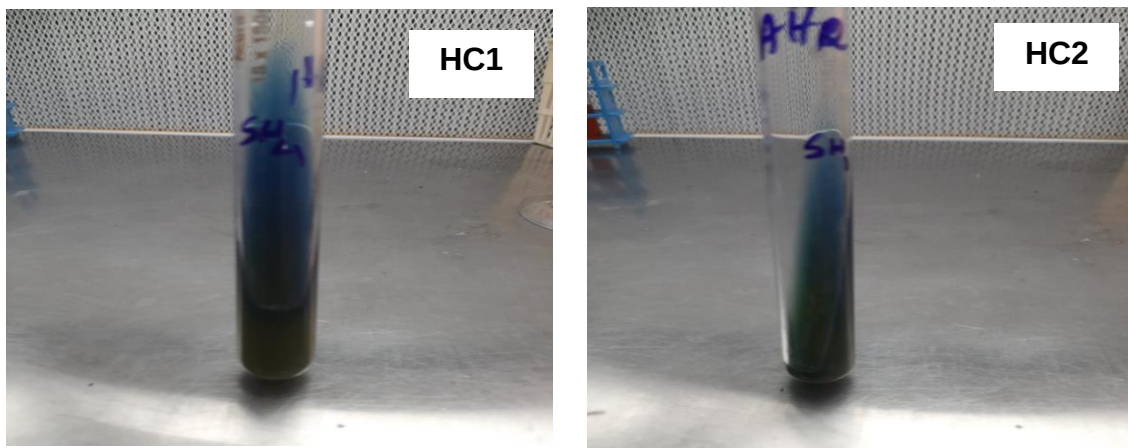


Fig. 5.3. Simmons Citrate Test

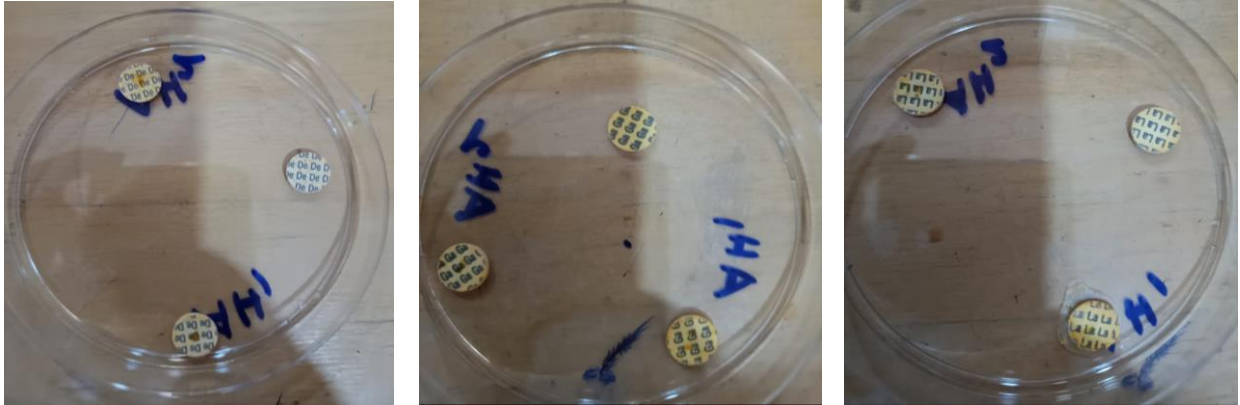


Fig. 5.4. Carbohydrate Test

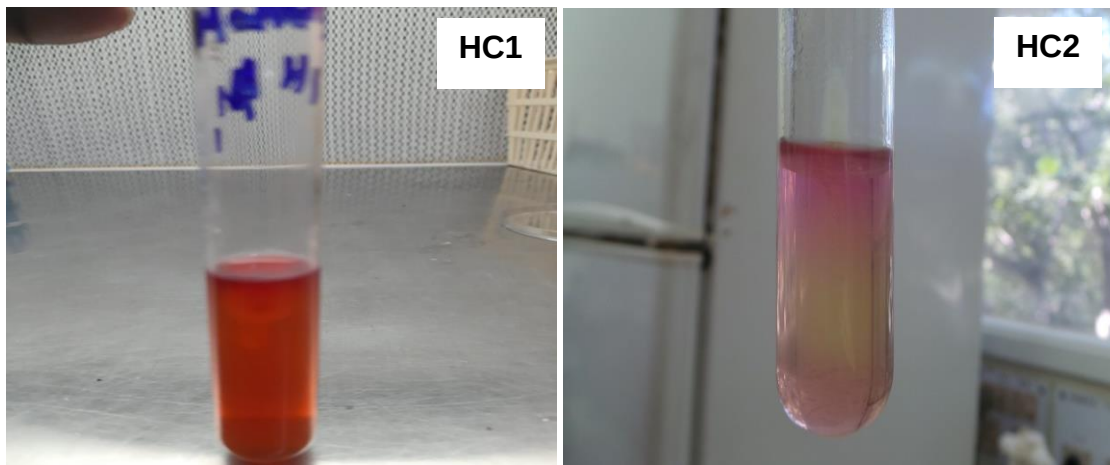


Fig. 5.5. Mannitol Motility Test

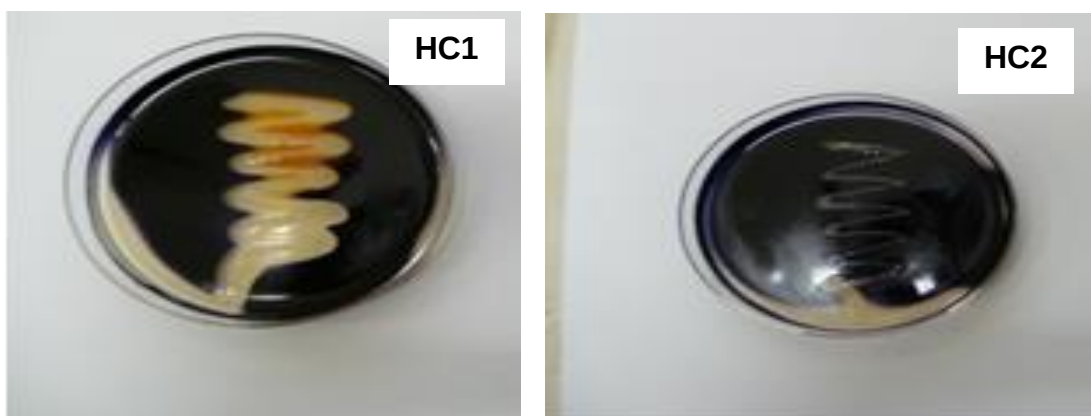


Fig. 5.6. Starch Hydrolysis Test

Table 5.2. Biochemical test results of unknown bacterial isolate

Tests	Hospital Culture (HC1)	Hospital Culture (HC2)
Gram Staining	Negative	Positive
Catalase	Positive	Positive
Starch hydrolysis	Positive	Negative
Citrate utilization	Positive	Positive
Mannitol motility	Positive	Positive
Dextrose	Positive	Positive
Lactose	Positive	Positive
Galactose	Positive	Positive
Identified Species	Pseudomonas sp.	Bacillus Sp.

5.2. Characterization of Nanoparticles using UV-Visible Spectroscopy

The presence of silver nanoparticle is confirmed from the spectra at 340, 373 and 424 nm³³. The presence of zinc oxide nanoparticle is confirmed from the spectra at 295 and 360 nm³⁴. It is further observed that the sharp peaks of Ag nanoparticles showed the effective separation of nano-Ag compared to the broaden peaks of nano-Zn synthesized from Aloe. However, there is no difference in the efficiency between these two nanoparticles.

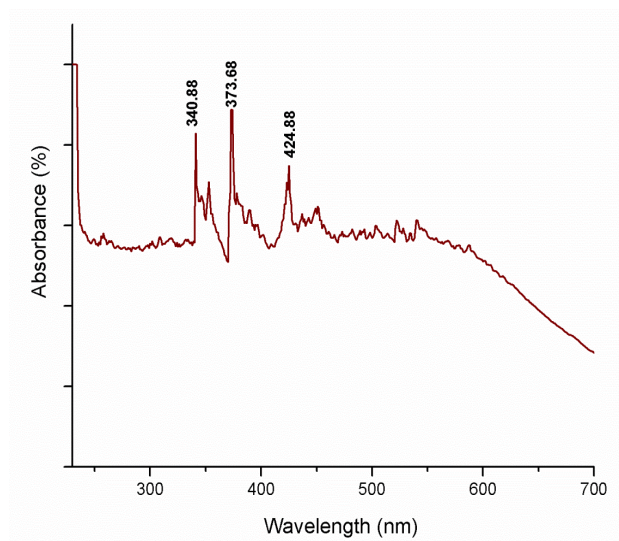


Fig. 5.7. Spectroscopic confirmation of Silver Nanoparticles from *Ocimum tenuiflorum*

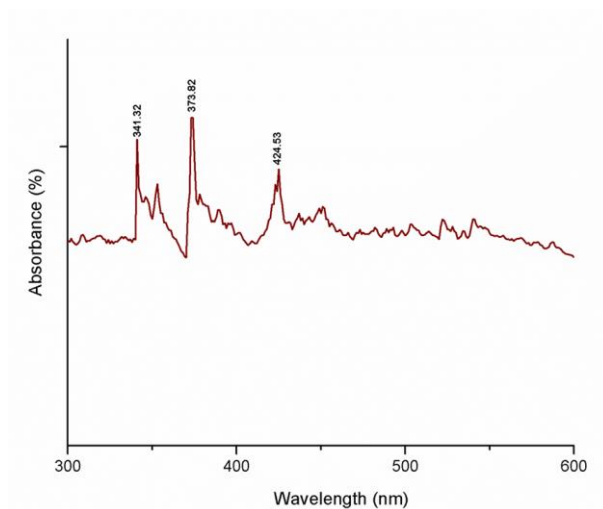


Fig. 5.8. Spectroscopic confirmation of Silver Nanoparticles from *Clitoria ternatea*

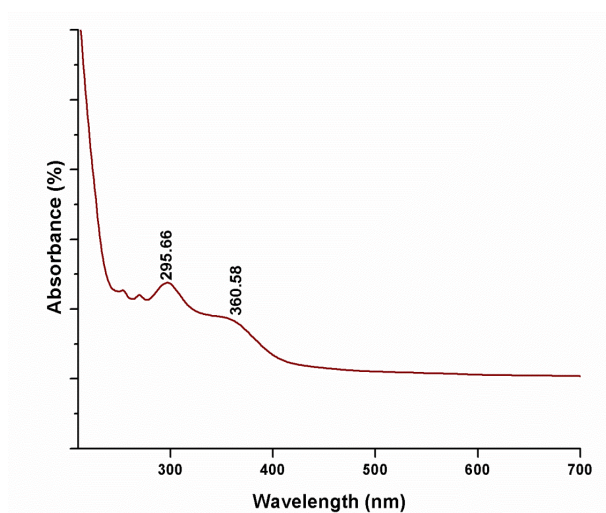


Fig. 5.9. Spectroscopic confirmation of Zinc Oxide Nanoparticles from *Aloe barbadensis miller*

5.3. Fluorescent microscopic images of corneal lenses coated with nanoparticles

The epifluorescent images of nanoparticles coated corneal lenses are shown in Figure 5.10 to 5.13. The orange red fluorescence rich surface of corneal lenses exposed to control culture confirming the more bacterial attachment compared to the nanoparticles coated corneal lenses.

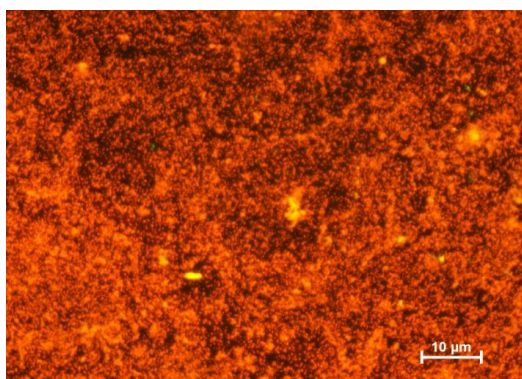


Fig.5.10. Culture – Nanoparticles (Control)

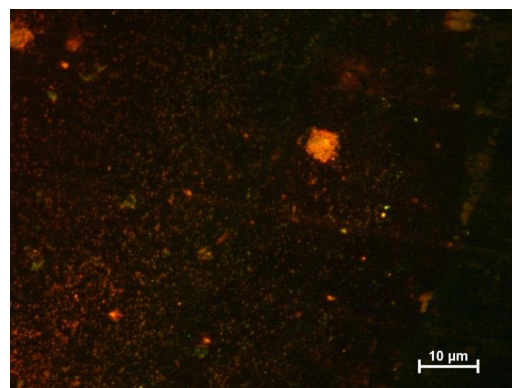


Fig.5.11. Culture + Zinc Oxide Nanoparticles from *Aloe barbadensis miller* Extract

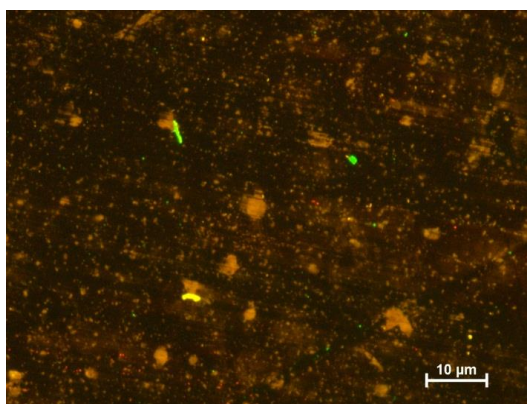


Fig.5.12. Culture + Silver Nanoparticles from *Clitoria ternatea* Extract

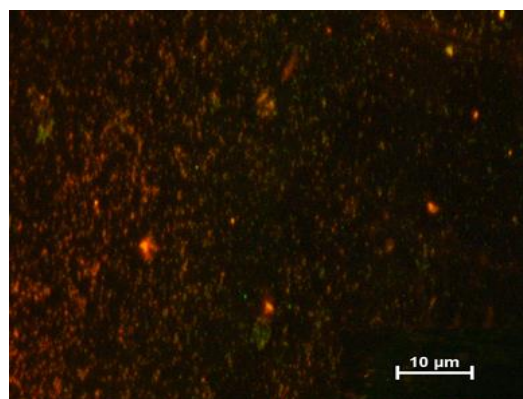


Fig.5.13. Culture + Silver Nanoparticles from *Ocimum tenuiflorum* Extract

The decreased orange fluorescence on the surface of corneal lenses exposed to nano particles admixed bacterial culture shows the inability of bacterial isolates to get adhere due to the disruption of cell membrane and generation of Reactive Oxygen Species (ROS). It can effectively stop the binary fission of bacterial cell membrane and there by no further bacterial proliferation.³⁴

6. SUMMARY AND CONCLUSION

Summary:

- Bacterial isolates scraped from the infected corneal lenses (Procured from Aravind Eye Hospital, Chennai) was plated (Pour plating method) and the most predominant single colonies were isolated and identified
- The plant extract of *Ocimum tenuiflorum* and *Clitoria ternatea*, and *Aloe barbadensis miller* was extracted and the nanoparticles are synthesized.
- The isolated nanoparticles are examined under UV-Vis Spectroscopy for the confirmation of its presence.
- The viable bacterial count was enumerated and the active and inactive cells were determined using BacTiter Glo and fluorescence microscope respectively.
- Results confirmed the improved anti-bacterial nature of nanoparticles derived from the plant extract.

Conclusion:

In order to extend the life span of corneal lenses which are used in a day-to-day life by the keratitis patients and when it is used for cosmetic purposes. Though nano-coated lenses are available in the market, those are costly and chemically-treated that are not prescribed for a healthy way of using it. Hence, in our study, it was proposed to coat the corneal lenses with nanoparticles which are synthesized from a natural way called green synthesis. The processes followed to fabricate green-aided nanoparticles coated corneal lenses are as follows.

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