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Efficacy of different therapeutic plant extracts and nanoparticles against pathogenic strains of *Streptococcus pneumoniae isolated* from ophthalmic infections

Running title: Antibacterial effect of therapeutic plants against *S. pneumonia*

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Abstract

Background: In recent years, eye infections are very common medical problem, which may be partly due to increasing in contact lens wearers. **Objective:** The objective of this study was to identify and characterize the microorganisms responsible for corneal infections. There is a significant public health concern regarding ophthalmic infections, particularly in environments that are contaminated. **Methodology:** Samples of eye swabs were collected from Fatima Memorial Hospital located in Shadman, Lahore. Samples were spread on Tryptic soy agar (TSA) and after that streaked on chocolate agar media for isolation of pure culture. After isolation of pure bacterial strains, pathogenicity of strains were checked by Blood agar test. Strains showed alpha hemolysis were choose and these strains were checked against different antibiotics, green synthesized silver Nano-particles of different plant extract and also against plant extract. **Results:** Four strains exhibited alpha hemolysis, indicating the presence of pathogenic strains of *Streptococcus pneumoniae*, which are implicated in ocular infections. Zone of inhibitions (ZOI) were

observed against different antibiotics, green synthesized silver Nano-particles of different plant extract and also against plant extract by some isolated bacterial strains but others showed resistance. **Conclusion:** The development of an effective and accessible treatment could contribute to global public health improvement. Accessible treatments may lead to better health outcomes, particularly in regions where ophthalmic infections are prevalent but access to advanced medical interventions is limited.

Keywords: Ophthalmic infections, *Streptococcus pneumonia*, Tryptic soy agar, Chocolate agar, Hemolysis, Antibiotic, Green synthesized silver nano-particles, Plant extract

Introduction:

Eye infections pose a considerable global health challenge, requiring immediate medical intervention and care [I].Among these infections, conjunctivitis is the most common in newborns, impacting approximately 2.6% to 8% of this age group [II]. The conjunctiva and eyelids are home to various microorganisms, including bacteria, viruses, and fungi from the surroundings, collectively termed normal flora. While these microorganisms can be advantageous by producing antibiotics and chemical mediators that aid in immunoregulation and homeostasis, they also compete for resources, which helps inhibit the growth of harmful bacteria. Although these organisms are generally harmless, certain circumstances can trigger them to cause infections [III]. It is crucial to recognize that both pathogenic and non-pathogenic bacteria exist in minimal amounts in the conjunctiva, primarily because most microbes are eliminated through tearing and blinking [IV].

Certain enzymes, including lysozyme, IgA, and IgG, exert a bacteriostatic effect by lowering the conjunctival temperature through tear evaporation, while the limited blood supply further restricts bacterial growth. The variety of microbes present on the conjunctiva differs among individuals due to factors such as genetic variations, sex, age, environmental influences, climate, and use of contact lenses, surgical history, antibiotic use, immune response, and geographic location [V]. Among various eye infections, red eye, corneal ulcer/keratitis, and conjunctivitis are significant conditions, ranking within the top five most common corneal infections. Conjunctivitis, an infection of the conjunctiva, occurs when blood vessels dilate, leading to inflammation [VI]. There are primarily three types of conjunctivitis: bacterial, viral, and fungal, with viral and bacterial forms being highly contagious and often resulting in red or pink eyes [VII]. Bacterial conjunctivitis is the most prevalent type, affecting

people from children to adults, but is more common in children [VIII]. Major pathogenic bacteria associated with bacterial conjunctivitis include *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* [IX].

A survey conducted in the USA in 1972 indicated that 13 out of every 1,000 individuals aged 1 to 70 develop conjunctivitis annually [X]. Viral conjunctivitis, primarily caused by the Herpes Simplex and Zoster viruses, is another widespread type of corneal infection, affecting adults more significantly than children [XI] Gonococcal conjunctivitis, the least common variant, is mainly seen in newborns and sexually active young adults [XII].

A common eye infection is microbial keratitis, which can rapidly impact the eye and demands immediate medical intervention. This form of corneal infection is often observed in adults, and without timely treatment, it may result in blindness. In the United States, microbial keratitis is the most frequently diagnosed eye infection, with nearly 30,000 cases reported annually [XIII]. Several factors increase the risk of this infection, such as contact lens usage, irregularities in the cornea, refractive surgery, diabetes, and conditions that impair the immune system. Furthermore, the types of microorganisms present can vary with climate and geographic factors, which are also important risk considerations for patients [XIV]. Bacterial keratitis is usually caused by a variety of bacteria, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, coagulase-negative *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Note *that Pseudomonas aeruginosa* is the most common bacteria that causes eye infections, especially in people who wear contact lenses. [XV].

Typical symptoms of bacterial keratitis include blurred vision, severe pain, light sensitivity, corneal opacity, and the presence of hypopyon (pus in the conjunctiva). Contact lens wearers may also notice redness or a pinkish tint in their eyes. While conjunctivitis is highly contagious, it can lead to vision loss if not treated swiftly [XVI].

Several viruses, such as Herpes Simplex, are known to cause keratitis in the USA, affecting different layers of the cornea, including the epithelial, endothelial, and stromal layers. Epithelial herpes simplex keratitis is characterized by dendritic ulcers and can be assessed using fluorescein staining and cobalt blue light [XVII]. In contrast, stromal herpes simplex keratitis can cause swelling and opacification of

the stroma, which can lead to ulcers, scarring, and blood vessel ingrowth. Endothelial keratitis affects the endothelial layer and may lead to corneal swelling and keratotic precipitates [XVIII].

Endophthalmitis is a severe eye infection caused by various microorganisms, including bacteria and fungi, resulting in inflammation. Although rare, this condition can quickly become an emergency, requiring immediate treatment to avert vision loss [XIX]. Despite the infrequency of keratitis and endophthalmitis, they present significant risks for visual impairment. Fungal infections and Acanthamoeba are the least common agents responsible for eye infections; fungal infections often arise in rural areas, while Acanthamoeba can lead to infections during swimming. Both types of infections can be effectively treated with antifungal and anti-Acanthamoeba medications [XX].

The most common pathogen linked to bacterial keratitis in contact lens users is Pseudomonas aeruginosa, which can lead to vision impairment. This bacterium is recognized as an extracellular pathogen, adhering to host tissues and releasing harmful substances that damage underlying tissues, resulting in erosive disease [XXI]. Other bacterial species, including *Salmonella*, *Shigella*, *Yersinia*, and various *Neisseria* species, can invade host cells and replicate within the cytoplasm or vacuoles of those cells [XXII].

Contact lenses are extensively utilized for aesthetic, corrective, and lifestyle reasons, with approximately 140 million people around the globe relying on them [XXIII]. Nevertheless, these lenses can carry significant drawbacks, such as the risk of ocular complications and, in some cases, vision loss. Contributing factors that can worsen vision and lead to ocular issues include extended wear, poor hand hygiene, and inadequate maintenance of lens cases. Such elements can promote bacterial adherence to the conjunctiva and disrupt the protective mucin layer, making it easier for bacteria to colonize [XIV].

Smartphones, particularly in clinical settings, can accumulate pathogens on their touchscreens, which may unintentionally be transferred to the eyes through touching or rubbing. Studies show that the pathogens present on contact lenses often match those responsible for corneal infections, resulting in corneal ulcers for those affected [XV].

Methodology:

Eye disease samples were collected using sterile culture at Shadman Fatima Memorial Hospital, Lahore. The samples were transported to the microbiology laboratory of Government College University, Lahore

where they were plated on agar plates.

Tryptic soy agar was utilized as the foundational medium for the isolation of pure cultures of pathogenic bacteria. After autoclaving, the TSA media was poured into petri dishes under laminar flow cabinet and let it to solidify. Once solidified, samples were distributed evenly over the TSA agar plates using culture sticks and a glass spreader. The petri dishes were incubated at 37°C overnight.

Bacterial colonies were picked from the TSA agar plates using an inoculating loop and streaked onto chocolate agar plates. These streaked plates were then incubated overnight at 37°C.

Nutrient agar served as the culture medium for these samples. To prepare it, 28 grams of nutrient agar were dissolved in 100 milliliters of distilled water, and the resulting solution was autoclaved at 131°C for 60 minutes.

Blood Agar Test was performed to measure the pathogenicity of different bacterial species. [XVI]. Nutrient agar media was prepared and autoclaved. After sterilization, as the temperature of media became normal non-coagulated blood was added into nutrient media and mix well. The medium was then poured into petri plates and let it to solidify. After solidification, the plates were streaked and incubated overnight at 37°C. A clear zone surrounding a colony indicates beta hemolysis, which signifies a positive result. In contrast, a greenish zone around a colony indicates alpha hemolysis, while the absence of clearing zones signifies gamma hemolysis, indicating no hemolytic activity.

To preserve bacterial cultures, a glycerol stock was prepared. Initially, 3 grams of nutrient broth were dissolved in 100 milliliters of distilled water and autoclaved at 131°C for one hour, then allowed to cool. An inoculating loop was utilized to introduce the bacteria into the nutrient broth, which was subsequently incubated overnight at 37°C to facilitate bacterial growth. Following incubation, 200 µl of glycerol was added to an Eppendorf tube, along with 800 µl of the nutrient broth containing the bacteria, and the mixture was vortexed thoroughly. Two sets of four bacterial strains were generated as glycerol stocks and stored the bacterial stock culture at -20°C in a freezer.

Antibiotic Activity against Isolated Bacterial Strains:

To assess antibiotic activity, plates of nutrient agar were prepared. Once the media into the plates get solid, the isolated bacterial strains were evenly spread on the plates using a sterilized spreader. Using sterile forceps, place two different antibiotics (Meronem and Cifixime) on the agar plate. Also, seven

antibiotic solutiones were prepared named amoxicillin, ceftriaxone, chloramphenicol, ciprofloxacin, oxytetracycline, doxycycline and azithromycin in a ratio of 5 g to 25 ml. Add approximately 5 µl of each antibiotic to the specific water of the petri dish.

The plates were then incubated overnight at 37ºC, and the bacterial strains' sensitivity to the antibiotic discs and solutions was evaluated by observing the clear zones of inhibition surrounding them. The diameters of these zones were measured with a ruler, and any bacterial growth near the antibiotic disc indicated resistance to the corresponding antibiotic.

Nanoparticle Activity against Isolated Bacterial Strains:

For the evaluation of nanoparticle activity against bacterial strains, plates of nutrient agar were prepared. Once the media into the plates get solid, the isolated bacterial strains were evenly spread on the plates using a sterilized spreader. Approximately 5 microliters of plant nanoparticles from species such as *Azadirachta indica, Cassia fistula, Calotropis procera,* and *Eucalyptus radiata* were introduced into the wells of the petri plates, which were then incubated overnight at 37ºC. The susceptibility of the bacterial strains to these nanoparticles was assessed by measuring the clear zones around the solutions using a ruler. Some strains exhibited growth around the nanoparticle solutions, indicating a level of resistance.

Plant Extract Activity against Isolated Bacterial Strains:

To examine the activity of plant extracts against isolated bacterial strains, 3 grams of nutrient agar were dissolved in 100 milliliters of distilled water and autoclaved for one hour at 121ºC. The prepared solution was subsequently poured into four sterilized petri plates under laminar flow conditions and allowed to solidify. Once set, the isolated bacterial culture was evenly spread across the plates with a sterilized glass spreader. Approximately 5 microliters of plant extracts from *Azadirachta indica*, *Cassia fistula*, *Calotropis procera*, and *Eucalyptus radiata* were added to the wells in the petri plates, which were then incubated overnight at 37ºC. The sensitivity of the bacterial strains to the plant extracts was evaluated by observing the clear zones formed around the solutions, which were measured with a scale. Some strains showed growth around the plant extract solutions, indicating their resistance to those extracts.

Results:

This study investigates bacterial eye infections. A variety of pathogens have been isolated from patients with conjunctivitis, and four distinct bacterial strains were examined.

Blood Agar Test for Bacterial Strains:

Strain 1, Strain 2, Strain 3 and Strain 4 isolates all showed complete α-hemolysis. A green halo around the bacterial strain indicated the presence of *S. pneumoniae*.

Effect of antibiotics against bacterial strains by disc diffusion method:

The zone of inhibition values ± S.E for strains 1 through 4 in response to the antibiotic disc Meronem were as follows: 8.25±0.478714 for strain 1, 7.75±0.478714 for strain 2, 6.5±0.288675 for strain 3, and 8.25±0.478714 for strain 4. Both strains 1 and 4 demonstrated the same sensitivity level to Meronem, while strain 2 was also susceptible to it; however, strain 3 was not. Conversely, the zone of inhibition values ± S.E for Cefixime across all isolated bacterial strains were recorded as zero, indicating that all strains showed resistance to Cefixime.

Effect of antibiotics against bacterial strains by Well Diffusion Method:

Seven different antibiotics were evaluated for susceptibility against the four isolated strains. The ± S.E. value for oxytetracycline among strains 1-4 was 10±0, suggesting moderate susceptibility across all strains. For doxycycline, the ± S.E. values for strains 1-4 were 15±2.886751, 11.25±1.652019, 9.5±0.5, and 9±1, respectively. Strain 1 displayed the highest sensitivity to doxycycline, while strains 2, 3, and 4 showed comparable susceptibility. The ± S.E. values for ciprofloxacin across strains 1-4 were 11.25±1.25, 5.75±0.853913, 12.75±1.314978, and 1.75±0.478714, respectively. Strain 3 was the most susceptible, while strain 4 was the least; strains 1 and 2 had similar susceptibility levels, with strain 2 exhibiting moderate susceptibility compared to the others. For chloramphenicol, the ± S.E. values for strains 1-4 were 9.5±0.5, 9.5±0.5, 9.5±0.5, and 4±1.581139, indicating that strains 1, 2, and 3 had the same susceptibility level, while strain 4 was less susceptible. Resistance to ceftriaxone was found in strains 1, 2, and 4, with strain 3 displaying a value of 2.75±0 S.E., indicating the largest zone of inhibition. For amoxicillin, the ± S.E. values were 9.5±0.5 for strain 1, 1.75±0.478714 for strain 3, and 1.5±0.478714 for strain 4, with strain 1 demonstrating the largest zone of inhibition, while strain 2 was completely resistant. Strains 2 and 4 were resistant to azithromycin, whereas strains 1 and 3 exhibited ± S.E. values of 3.25±1.652019 and 1.75±0.478714, respectively, indicating that strain 1 was more

susceptible to azithromycin. Tukey's and Bonferroni's statistical analyses revealed significant differences among the responses to oxytetracycline, azithromycin, and ceftriaxone across the bacterial strains, underscoring varying levels of resistance. The p-value for oxytetracycline was less than 0.05, indicating a significant difference in responses among the bacterial strains compared to ceftriaxone, amoxicillin, and azomax, with notable differences also observed between ciprofloxacin and ceftriaxone.

Effect of antibiotics against bacterial strains against silver green synthesized nanoparticles:

The standard error (±S.E) values for *Cassia fistula* in strains 1 to 4 are measured at 4±0.91, 4.75±0.87, 3±0.82, and 4.25±0.48, respectively. Among these, Strain 2 demonstrated the highest sensitivity. In the case of *Azadirachta indica*, the zone of inhibition values for the strains are 4.5±2.87, 3.25±2.25, 5±1.58, and 4.5±1.04, with Strains 1 and 4 displaying the greatest susceptibility overall. The ±S.E values for *Eucalyptus radiata* in strains 1 to 3 are recorded as 3.5±1.55, 4.75±1.70, and 5.5±1.76, respectively. Here, Strain 3 indicates the highest sensitivity, while Strain 4 exhibited resistance to *Eucalyptus radiata*. For *Calotropis procera*, the inhibition zone values for strains 1 to 4 are 1.75±0.48, 4.5±2.02, 2.75±1.11, and 3.5±1.19, with Strain 4 demonstrating the most marked sensitivity among the tested strains. All bacterial strains showed a greater P value, implying comparable antibacterial activity as determined by Tukey and Bonferroni tests. Therefore, these strains are statistically significant.

Effect of antibiotics against bacterial strains against Plant Extracts:

The standard error (S.E.) values for *Azadirachta indica* in strains 1 to 4 are as follows: 6±1.47, 8.25±0.63, 7.5±2.12, and 10.25±1.11. Strain 4 exhibited the highest susceptibility to *Azadirachta indica* relative to the other strains. For Eucalyptus radiata, the zone of inhibition values for strains 1 to 4 are recorded as 5.5±0.87, 8.25±0.48, 9±2.5, and 10.25±1.11, with Strain 4 showing greater sensitivity than the others. All strains demonstrated resistance to *Calotropis procera* and *Cassia fistula*.

Table 1: Types of Hemolysis for isolated bacterial strains

Isolated Bacterial Strains	Pathogenicity	Type of Hemolysis
Strain 1	Positive	Alpha Hemolysis
Strain 2	Positive	Alpha Hemolysis
Strain 3	Positive	Alpha Hemolysis
Strain 4	Positive	Alpha Hemolysis

Table 2: Mean± S.E value of antibiotic against bacterial strains by disc diffusion method

Isolated Bacterial Strains	Inhibition Zones (mm)	
	Meronem	**Cefixime**
Strain 1	8.25±0.478714	Resistant
Strain 2	7.75±0.478714	Resistant
Strain 3	6.5±0.288675	Resistant
Strain 4	8.25±0.478714	Resistant

Table 3: Mean ± S.E value of antibiotic against bacterial strains by well diffusion method

	Zone of inhibition (mm)			
Antibiotics	**Strain 1**	**Strain 2**	**Strain 3**	**Strain 4**
Oxytetracycline	10±0	10±0	10±0	10±0
Doxycycline	15±2.886751	11.25±1.652019	9.5±0.5	9±1
Ciprofloxacin	11.25±1.25	5.75±0.853913	12.75±1.314978	1.75±0.478714
Chloramphenicol	9.5±0.5	9.5±0.5	9.5±0.5	4±1.581139
Ceftriaxone	Resistant	Resistant	2.75±0	Resistant
Amoxiciline	9.5±0.5	Resistant	1.75±0.478714	1.5±0.478714
Azithromycine	3.25±1.652019	Resistant	1.75±0.478714	Resistant

Table 4: Antibacterial activity of silver green synthesized nanoparticles isolated against bacterial strains

	Inhibition Zones (mm)			
Isolated Bacterial Strains	***Cassia fistula***	***Azadirachta indica***	***Eucalyptus radiate***	***Calotropis procera***
Strain 1	4±0.912878	4.5±2.872281	3.5±1.554563	1.75±0.478714
Strain 2	4.75±0.87459	3.25±2.25	4.75±1.701715	4.5±2.020726
Strain 3	3±0.816497	5±1.581139	5.5±1.755942	2.75±1.108678
Strain 4	4.25±0.478714	4.5±1.040833	Resistant	3.5±1.190238

Table 5: Antibacterial activity of plant extracts against isolated bacterial strains

Strains Isolated Bacterial Strains	Zone of inhibition(mm) SNPs of *Azadirachta indica*	Zone of inhibition(mm) SNPs of *Eucalyptus radiata*	SNPs of *Calotropis procera*	SNPs of *Cassia fistula*
Strain 1	6±1.47196	8.5±0.866025	Resistant	Resistant
Strain 2	8.25±0.629153	8.25±0.478714	Resistant	Resistant
Strain 3	7.5±2.12132	9±2.5	Resistant	Resistant
Strain 4	10.25±1.108678	10.25±1.10867	Resistant	Resistant

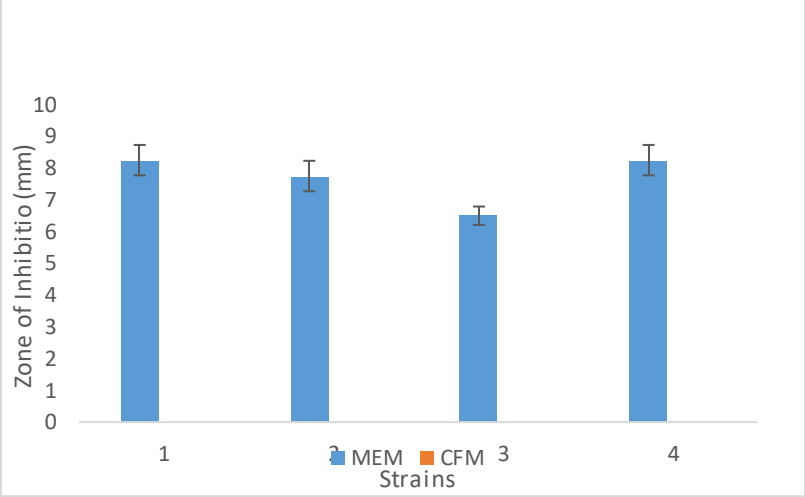

Figure. 1: Antibacterial activity against antibiotic discs

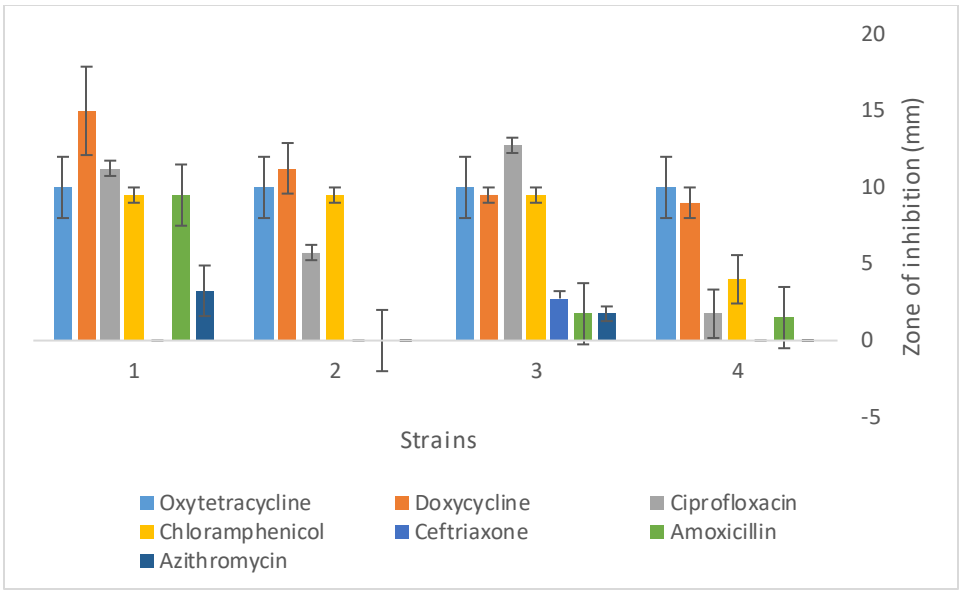

Figure. 2: Antibacterial activity of antibiotics by well diffusion method

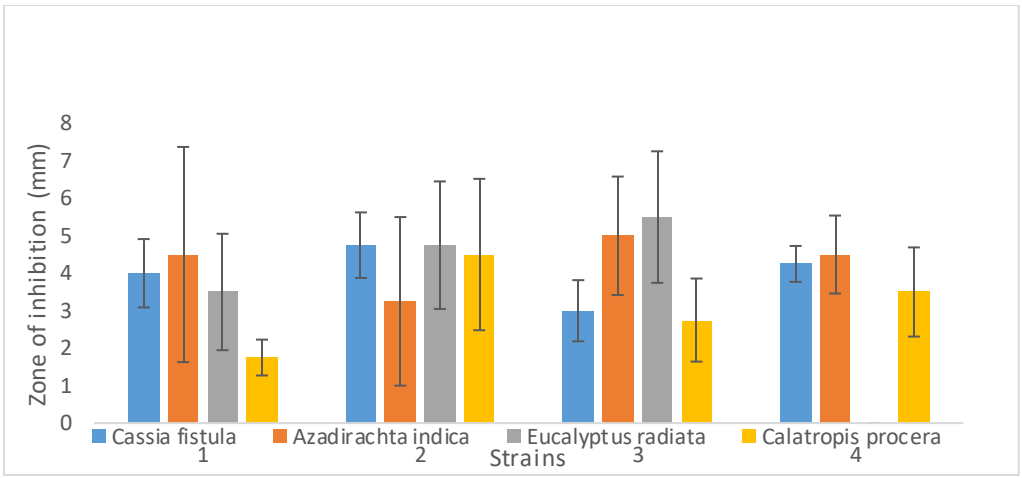

Figure. 3: Antibacterial activity of green synthesized silver nanoparticles

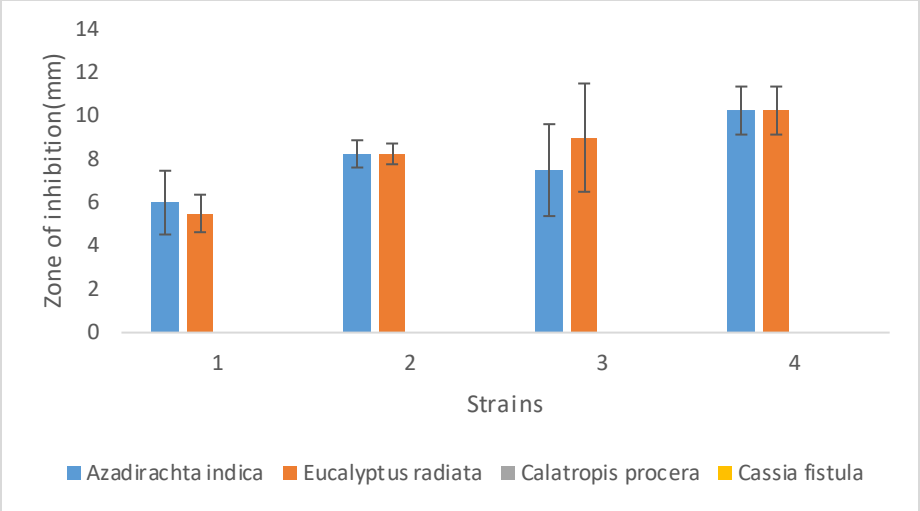

Figure. 4: Antibacterial activity of Plant extract

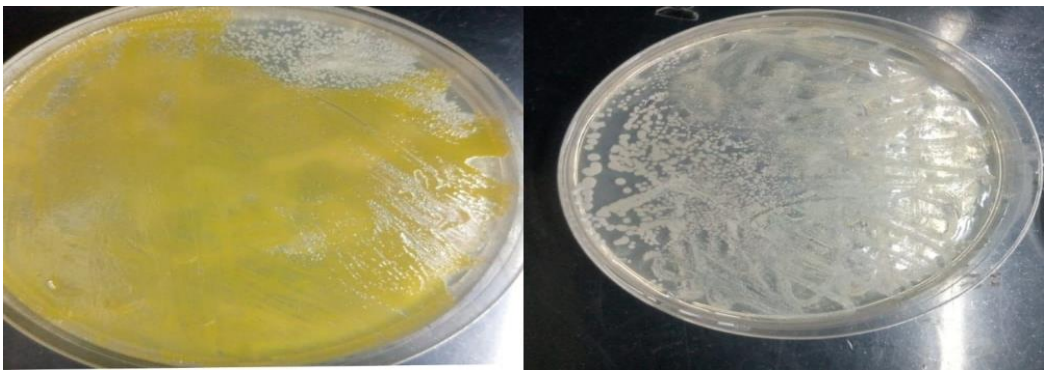

Figure. 5: Spreading of sample on TSA

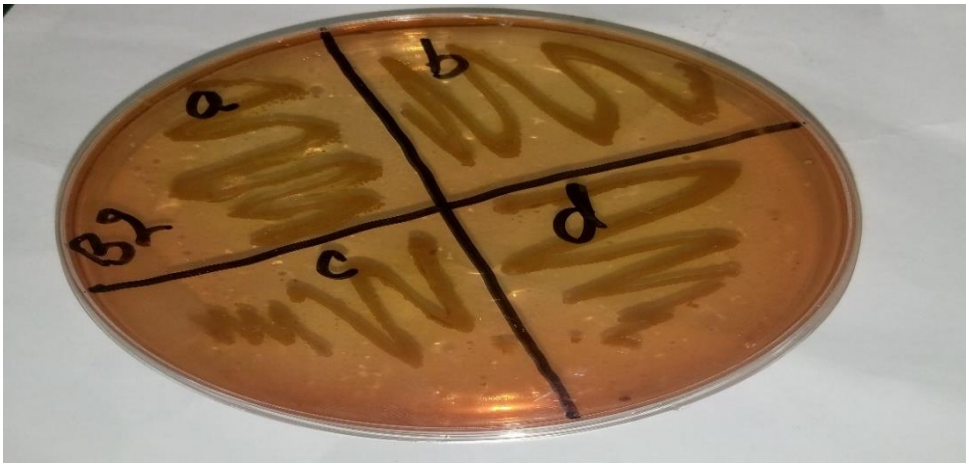

Figure. 6: Pathogenicity test

Figure. 7: Antibacterial activity by disc diffusion method

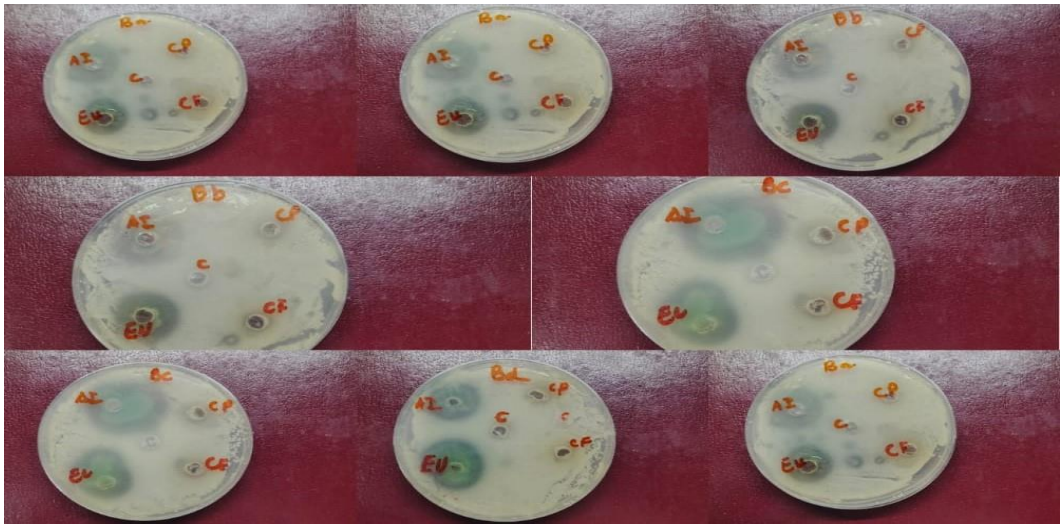

Figure. 8: Antibacterial activity of SNPs Plant extract against bacterial strains

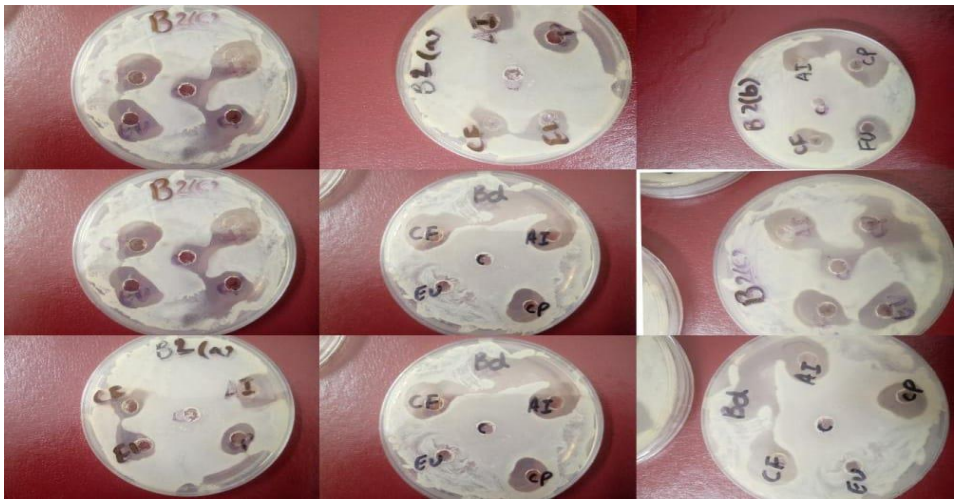

Figure. 9: Antibiotic activity against bacterial strains

DISCUSSION

Bacterial pathogens usually affect the eye's outer surface while leaving the internal structures untouched, as the latter are maintained in a sterile environment. Numerous immunological mechanisms are active within the internal eye. Several factors can jeopardize eye health, including injury to the epithelial layer, compromised systemic immunity, and pathogens from other areas of the body. The conjunctiva has multiple defense strategies, including immune cells, fibronectin, immunoglobulins, and lysozyme, all of which play a role in fighting off pathogens. Weakened immune responses can lead to bacterial infections, which can subsequently be addressed with a range of antibiotics [XVII].

This study concentrated on ophthalmic infections and their vulnerability to various antibiotics used for treatment. Antibiotic discs were applied to bacterial strains to evaluate their susceptibility, revealing that Cefixime demonstrated complete resistance across all bacterial strains tested. Research indicates a 91.4% resistance rate for cefixime concerning bacterial infections, with only 6.2% susceptibility noted among gram-positive strains [XVIII]. Meropenem produced the largest zones of inhibition, measuring between 8.25 mm and 6.25 mm, and exhibited a 9.09% resistance rate against isolated strains, marking it as an effective antibiotic for ophthalmic infections [XIX].

Seven antibiotics; oxytetracycline, doxycycline, Azomax, ciprofloxacin, chloramphenicol, amoxicillin, and ceftriaxone—were evaluated against four isolated bacterial strains. The p-value for Azomax, oxytetracycline, and ciprofloxacin was below 0.05, with ciprofloxacin showing the most significant

inhibitory effect, reflected by a zone of inhibition of 12.25 mm. Ceftriaxone was resistant against strains 1, 2, and 4, while strain 2 also exhibited resistance to amoxicillin and azithromycin.

One-way ANOVA analysis indicated significant differences among the antibiotic dilutions, supported by a p-value of less than 0.05. To pinpoint the sources of these significant differences, post-hoc evaluations using Tukey's and Bonferroni tests were performed, revealing that nearly all antibiotic dilutions exhibited comparable antibacterial activity except for ciprofloxacin, which stood out with notably superior antibacterial performance.

A direct relationship exists between the resistance of bacterial pathogens and antibiotics, primarily resulting from the overuse of antibiotics for both systemic and topical infections. Additional factors contributing to increased resistance include prolonged treatment, migration, and non-bacterial infections [XXX]. On the other hand, some antibiotics have displayed varying susceptibility levels against bacterial strains. Tetracycline and chloramphenicol, both bacteriostatic agents, inhibit protein synthesis. While chloramphenicol is commonly used for various bacterial infections in Asian nations, it is prohibited in the United States due to its detrimental effects on bone marrow. Nevertheless, both antibiotics can still be employed to treat ophthalmic infections [XXXI].

Plants are known for their therapeutic properties and can effectively address various diseases, including bacterial infections, with considerable medical implications. In this investigation, various plant extracts and silver nanoparticles (SNPs) sourced from plants were utilized to evaluate their antibacterial effectiveness [XXXII]. Antibacterial evaluations of SNPs from *Eucalyptus radiata*, *Cassia fistula*, *Calotropis procera*, and *Azadirachta indica* were performed against isolated bacterial strains, with only strain 4 showing complete resistance to *Eucalyptus radiate* [XXXIII]. Treatment of bacterial strains with plant extracts revealed that *Calotropis procera* and *Cassia fistula* exhibited complete resistance against the strains [XXXIV]. However, *Azadirachta indica* and *Eucalyptus radiata* displayed the highest zones of inhibition at 10.25 mm. *Azadirachta indica* is recognized as an effective herbal remedy for *E. coli*, *P. aeruginosa*, *S. aureus*, and *P. pyogenes*, especially under specific conditions such as optimal temperature and pH levels [XXXV, XXXVI].

Ethical Approval

The study was approved by Board of studies (BOS), department of Zoology and Advance Studies and Research Board, Government College University Lahore (REG-ACAD-ASRB/57/24/022). Also approved by ethical committee of institution.

Declarations

All authors listed in paper have made important contributions and there is no conflict of interest among authors.

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