https://doi.org/10.48047/AFJBS.7.8.2025.296-316

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Screening and molecular characterization of bacterial pathogens from river Ravi water used for irrigation purpose and role of biological antibacterial agents against bacterial isolates

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Volume:7,Issue,8Aug2025
Received:15Jun,2025
Accepted:22July,2025
Published:01Aug2025

doi:10.48047/AFJBS.7.8.2025.296-316

Abstract

Two bacterial strains were isolated from water samples. To check the pathogenicity of isolates, blood agar test was performed. Both isolated bacterial strains showed β hemolysis. Antibiotic susceptibility/resistance test was performed to check the resistance of isolated bacterial strains against different antibiotics. Cefixime, Meronem, Doxycycline, Oxytetracycline, Chloramphenicol, Ciprofloxacin, Amoxicillin and Azomax were used to check antibiotic resistance or susceptibility of isolated bacterial strains. Strain 1 showed resistance against cefixime and strain 2 showed resistance against ceftriaxone. Green synthesized silver nanoparticles and plant extracts of Neem (*Azadirachta indica*), *Calotropis procera*, *Cassia fistula* and *Eucalyptus radiata* were also used to check the antibacterial activity of isolated bacterial strains. No strain showed resistance against any naonoparticle. Both strains did not show any antibacterial activity against aqueous plant extracts. Molecular characterization or ribotyping results showed that isolated bacterial strains were *Escherichia coli* and *Enterococcus* species. The study showed that river Ravi water is suitable for irrigation purpose due to presence of different pathogenic microbes in river water and ultimately cause different infections in humans. Due to overuse of antibiotics bacteria developed resistance against some antibiotics so nanoparticles and plant extracts should be used as antibacterial tools against which bacteria might not developed resistance.

Key words: River Ravi, Bacterial Pathogens, Antibiotic resistance, Molecular characterization, Therapeutic plants

Introduction/Background

Water is necessary for every living organism. Unfortunately, many people do not have access to safe drinking water mostly in developing countries or in some region of developed countries. Due to unimproved sanitation two and a half billion people affected worldwide and more than 1.5 million children died every year due to gastrointestinal infections (Fenwick, 2006 and Hinrichsen and Tacio, 2002).

Water borne diseases are transmitted through the ingestion of water that is contaminated due to different pathogens. The infections caused by different pathogenic microbes depend on different factors like survival and division or multiplication of pathogens in the environment. Different chemical factories or industries during manufacturing of different products released harmful substances that are discharged into fresh water reservoirs like lakes, rivers *etc.* and polluting different water bodies (Bill, 2010 and Cabral, 2010). Pollutants that are released from factories and industries must be treated through separate waste plant systems (Maczulak, 2010 and Pope III *et al*., 2016). Water borne bacterial pathogens are classified into enteric and aquatic bacteria. Enteric bacteria such as *E. coli*, *Salmonella* spp. or *Shigella* spp. are present in the intestines of humans and some can also grow in freshwater bodies like rivers, lakes and streams. Presence of enteric bacteria in water depends on some parameters like temperature and nutrient availability. Aquatic bacteria like *Aeromonas* spp. *Pseudomonas aeruginosa* and *Mycobacterium avium* are present in water and grow or multiply in the drinking water. In US estimated 1200 deaths reported due to the waterborne infections (Foca *et al*., 2000).

From different industrial or agricultural industries wastes are direct disposed into rivers without any treatment and can change the biological, chemical and physical parameters of surface water resources. Surface water becomes inappropriate for irrigation, drinking and aquatic life and cause serious waterborne diseases (Kanu and Achi, 2011). Coliforms fecal bacteria enter in rivers through direct disposal of wastes or through runoff from areas where greater amount of animals present. Runoff from septic tanks, agriculture industries, sewage plant can increase the coliforms and bacterial pollution in rivers. Sewage may be transferred into rivers through heavy rain falls (Joshi *et al*., 2016).

River Ravi that starts flowing from India and ends in Pakistan irrigates many agricultural lands but unfortunately due to different anthropogenic activities like sewage dumping and animal feces river Ravi contaminated and become menace for aquatic life and terrestrial life due to presence of pathogenic bacteria. In this study microbial quality of river Ravi water near Shahdara evaluated by characterization and screening of pathogenic bacteria through microbiological techniques (Islam *et al*., 2017).

Misuse or overuse of antibiotics increase the resistance in different bacteria (Grenni *et al*., 2018 and Laxminarayan, 2014). Gram negative bacteria like *Shigella*, *Salmonella*, *E.coli* are multi drug resistant in comparison with gram positive bacteria due to the presence of an outer membrane that may act as permeability barrier and preventing different antibiotics to penetrate into the cells but Gram positive bacteria lacks outer membrane (Sharma *et al*., 2017). Large amount of antibiotic resistant genes are present in the environment including surface water rivers, lakes, stream due to livestock waste discharges, sewage and industrial and hospitals effluents and wastewater treatment plants (He *et al*., 2020 and Rizzo *et al*., 2013). In wastewater treatment plants (WWTPs) antibiotic resistant genes are present because they are unable to eliminate antibiotics and not designed for this purpose (Yang *et al*., 2020).

Modes of action of nanoparticles are different from antibiotic resistance genes (Bruna *et al*., 2021). Silver nanoparticles are known for its antibacterial and antifungal effects and have been used against different bacterial infections. Silver nanoparticles have antibacterial activity against different gram positive, gram negative and multi drug resistant bacteria. Silver nanoparticles due to its antibacterial effect and different unique mode of actions show synergistic effect against different pathogenic bacteria like *E.coli* and *Staphylococcus aureus*. Silver nanoparticles show unique characteristics like have ability to penetrate deep into the cell wall, changed the cell membrane structure and integrity and exhibit high surface to volume ratio. These characteristics of silver nanoparticles make them suitable to use in different medicines and to prevent different bacterial infections (Saeb *et al*., 2014). Green synthesis of silver nanoparticles by using different plant extracts is an alternative of chemical method and this method is environmental friendly and cost effective. Green synthesis production of silver nanoparticles is widely used in many applications of different industries, and biomedicines. Due to the presence of different naturally present biomolecules like proteins, carbohydrates, alkaloids and vitamins in plants used in formation of silver nanoparticles (Loo *et al*., 2018).

Due to increase resistance in bacteria against different antibiotics different plants and herbs are used as an alternative for antimicrobial activity. Plants were used in Chinese, Unani and Ayurveda medicines since ancient times due to their beneficial remedies (Talib and Mahasneh, 2010). Among all discovered plant species only one to ten percent is used for antimicrobial activity. Medicinal plants have been used as remedy for the antibacterial activity after many failures of antibiotics against different multi drug resistant bacteria (Abdallah, 2011 and Petti *et al*., 2005).

The aim of this research is characterization and screening of pathogenic bacteria present in river Ravi water that is used for irrigation purpose and find out about susceptibility or resistance of isolated bacterial strains against different antibiotics, green synthesized nanoparticles and plant extracts.

Materials and Methods

Collection of samples

Water samples were collected from river Ravi near Shahdara in sterilized caped bottles to isolate the pathogenic bacteria and evaluate the microbial quality of river water. Water samples were carried at Government College University Lahore and stored at 4°c to do culturing of samples and run the samples prolong.

Preparing cultural plates:

Nutrient agar was used as medium to isolate the bacteria. It was prepared by adding 12 g nutrient agar into 500ml distilled water. Prepared medium was autoclaved at 15 Ib pressure for 30 minutes at 121°C temperature. Medium was poured into petri plates under laminar flow to avoid contamination and allowed it to solidify for 10-15 minutes. After this put the petri plates into incubator for 24 hours to check the contamination.

Isolation of bacteria from water samples (Spreading of water samples)

50 ul of water samples were spread on agar petri plates through spreader. To get the isolated pure bacterial colonies placed the petri plates into incubator for 24 hours (Figure 1).

Inoculation of pure culture

After 24 hours different bacterial colonies were appeared on agar plates. Streaked the isolated colonies through sterilized inoculated loop onto solidify autoclaved agar plates and placed the streaking plates into the incubator for 24 hours at 37° (Table 1) (Figure 2).

Glycerol stock preparation

Glycerol stock is prepared to preserve the isolated bacterial strains. Medium was prepared by adding 3g nutrient broth into 100 ml distilled water in a flask. Autoclave at 15Ib pressure for 30 minutes at 121°C temperature. Add 200ul glycerol in eppendorfs and autoclave at 15Ib pressure for 30 minutes at 121°C temperature. Bacterial cultures were grown in autoclaved nutrient broth containing falcon tubes by applying inoculum through use of sterilized inoculated loop. After this bacterial cultures were placed in incubator for 24 hours at 37°C. 800ul of cultural broth was added into 200ul of autoclaved glycerol containing eppendorf in the laminar flow and vortex for 10-20 seconds. Prepared the three sets of glycerol stock for each bacterial strains and refrigerated at 20°C

Blood agar test

To check the pathogenicity of isolated streaked bacterial colonies blood agar test was performed. Blood in the medium provides necessary nutrients and supplements that allow the growth of pathogenic bacteria and inhibit the growth of *Neisseria* and *Haemophillus* spp. To prepared the medium dissolve 3 g nutrient agar into 100 ml distilled water. Autoclaved the medium at 15 Ib at 121°C for 30 minutes. Cooled the medium until temperature reached to 45°C. Add the 3 ml non coagulated blood into medium at 45°C temperature. If blood is added at high temperature then it will convert into chocolate agar due to hemolysis of RBCs by heating blood agar changed into chocolate agar. On chocolate agar only few bacterial species like *Neisseria* and *Haemophillus* spp. were grown. After adding blood poured the medium into petri plates and allowed it to solidify for 10 minutes. From the isolated streaked bacterial colonies again streak onto the blood agar plates. Put the plates in incubator for 24 hours at 37°C. Appearance of growth showed positive results. Clear zone around streak showed beta hemolysis. Greenish zone around streak showed alpha hemolysis. No zone around streak showed gamma hemolysis which means bacterial strains were non-pathogenic (Table 2).

Antibiotic resistance of isolated bacterial strains (Disc diffusion method)

Disc diffusion method was used to check the antibiotic resistance of isolated bacterial strains. Two antibiotic discs cefixime and meronem were used to check the susceptibility of antibiotics against isolated bacterial strains. Medium was prepared by adding 3 g nutrient agar into 100 ml distilled water. Autoclave the medium at 15 Ib at 121°C for 30 minutes. Poured this media into four petri plates and allowed it to solidify for 10 minutes in the laminar flow. By using spreader spread the bacterial strains throughout the petri plates. Antibiotic discs were placed through forceps on petri plates. Put the agar plates into incubator for 24 hours at 37°C. Clear zone formation around bacterial strains called as zone of inhibition. Zone of inhibition measured by measuring scale in mm. No growth or no zone formations around antibiotic discs showed bacterial strains were resistant to antibiotics (Table 3).

Antibiotic resistance of isolated bacterial strains (Well diffusion method)

Well diffusion method was used to check the antibiotic resistance of isolated bacterial strains. Different antibiotics used and prepared at 5 mg/25 ml concentration. Water used as control group. Medium was prepared by adding 6 g nutrient agar into 200 ml distilled water. Autoclaved the medium at 15 Ib at 121°C for 30 minutes. Poured this medium into eight petri plates and allowed it to solidify for 10 minutes in the laminar flow. In four plates made five wells and spread the bacterial strains throughout the plate with spreader. Add 50 ul of four antibiotic solutions including doxycycline, oxytetracycline, ciproflloxacin, chloramphenicol into four wells and water as control in fifth well. Put the agar plates into incubator for 24 hours at 37°C. In next four plates made four wells and spread the bacterial strains throughout the plate with spreader. Add 50 ul of three antibiotic solutions including ceftriaxone, amoxicilin, azomax or azithromycin into three wells and water as control in fourth well. Put the agar plates into incubator for 24 hours at 37°C. Clear zone formation around bacterial strains called as zone of inhibition. Zone of inhibition measured by measuring scale in mm. Growth around antibiotic discs showed bacterial strains were resistant to antibiotics (Table 4).

Antibacterial activity test of Nanoparticles (Green Synthesis)

Antibacterial activity of various nanoparticles was tested through well diffusion method. Solution of nanoparticles was prepared by dissolving 0.0017g of silver nitrate $AgNO_3$ (10mM solution) into 100ml of deionized water. Plant extracts were taken in a burette and silver nitrate solution was added in it drop wise. Formation of nanoparticles was indicated by the change of color from colorless solution to brown color. Change in color was the indication of formation of nanoparticles solution. The change in color indicated that silver is reduced from Ag^+ to Ag^0. Four types of nanoparticles were used which include silver nanoparticles (SNPs) of *Azadirachta indica* (Neem), *Cassia fistula*, *Calotropis procera*, *Eucalyptus radiata*. Medium was prepared by adding 3 g nutrient agar into 100 ml distilled water in a flask. Autoclaved the medium at 121°C for 30 minutes at 15 Ib pressure. Medium was poured in four plates and allowed to solidify for 10 minutes. 5 wells were made in each plate and bacterial culture was spread evenly on each plate by spreader. 50 ul solution of nanoparticles were added in wells separately. Water added in one well as control. Placed the plates in incubator for 24 hours at 37°C. Zone of inhibition measured by measuring scale in mm. Zone of inhibition surrounding the wells indicated the bacterial strain sensitivity to a specific nanoparticle. Growth around wells showed resistance of isolated bacterial strains to specific nanoparticle (Table 5).

Antibacterial activity test of Plant Extracts

Antibacterial activity of various plant extracts was tested by well diffusion method. Plant extracts were synthesized by using leaves of *Neem*, *Calotropis procera*, *Cassia fistula* and *Eucalyptus radiata* and washed with water and cut into fine pieces. Pieces were washed with deionized water and air dried at room temperature. 20 ug leaves were poured into beaker containing 200 ml deionized water and were placed on magnetic stirrer for 10 minutes at 50-60 minutes. Extract was filtered with Whatman filter paper No. 1 and after this cooled it down. For each experiment fresh plant extract was prepared. Plant extracts of *Azadirachta indica* (Neem), *Cassia fistula*, *Calatropis procera*, *Eucalyptus* was used. Medium was prepared by adding 3 g nutrient agar into 100 ml distilled water in a flask. Autoclaved the medium at 121°C for 30 minutes at 15 Ib pressure. Medium was poured in four plates and allowed to solidify for 10 minutes. 5 wells were made in each plate and bacterial culture was spread evenly on each plate by spreader. 50 ul solution of nanoparticles were added in wells separately. Water added in one well as control.

Placed the plates in incubator for 24 hours at 37°C. Zone of inhibition measured by measuring scale in mm. Zone of inhibition surrounding the wells indicated the bacterial strain sensitivity to a specific plant extract. Growth around wells showed resistance of isolated bacterial strains to specific plant extract (Table 6).

Molecular characterization of isolated strains

DNA extraction of isolated strains was done by using Phenol chloroform extraction methodology and after PCR, sequencing of the PCR products was done. Sequence blast was done on NCBI for getting accession number of isolated strains.

RESULTS

Blood agar test of isolated bacterial strains

All isolated bacterial strains (Strain 1 and strain 2) showed beta hemolysis. Beta hemolysis means complete hemolysis of RBCs and appearance of transparent zone around isolated bacterial strains. Beta hemolysis in blood agar test indicated that isolated bacterial strains are pathogenic (Figure 4).

Antibiotic resistance of isolated bacterial strains ± S.E value

Zone of inhibition (mm) ± S.E value of strain 2 against cefixime was 2.75±0.25. No zone of inhibition around strain 1 showed resistance against cefixime. Zone of inhibition ± S.E value of strain 1 and 2 against meronem was 14±0, 10.25±0.63. Strain 1 showed more susceptibility against meronem and strain 3 showed less susceptibility against meronem.

Zone of inhibition ± S.E value of strain 1 and strain 2 against doxycycline was 12.25±0.85, 12.25±0.85. Zone of inhibition ± S.E value of strain 1 and strain 2 against oxytetracycline was 9.25±0.25, 4.75±0.25. Zone of inhibition ± S.E value of strain 1 and strain 2 against ciprofloxacin was 9.75±0.25, 7.5±0.25. Zone of inhibition ± S.E value of strain 1 and strain 2 against chloramphenicol was 5±0, 7.5±0.5. Zone of inhibition ± S.E value of strain 1 against ceftriaxone was 12.75±1.03. No zone of inhibition around strain 2 showed resistance against ceftriaxone. Zone of inhibition ± S.E value of strain 1 and strain 2 against amoxicillin was 11.25±0.48, 6.75±1.03. Zone of inhibition ± S.E value of strain 1 and strain 2 against azomax was 11.25±0.48, 10.75±0.25.

On way ANNOVA test applied to all antibiotics including doxycycline (DOX), oxytetracycline (TET), ciprofloxacin (CIP), chloramphenicol (CHL), ceftriaxone (CEF), amoxicillin (AMO) and azomax (AZ0). ANNOVA test applied and results showed p value was less than 0.05 ($P<0.05$). Significant difference between antibiotics checked with post-hoc analysis. Bonferroni and tuckey test was performed. Results showed that all antibiotics showed similar antibacterial activity except cefixime and ceftriaxone because they showed significant difference in antibacterial activity with all other antibiotic dilutions. Strain 1 showed resistance against cefixime and strain 2 showed resistance against ceftriaxone and showed less antibacterial activity than other antibiotics (Figures 3, 4, 5, 6).

Antibacterial activity test of Nano particles ± S.E value

Zone of inhibition (mm) ± S.E value of strain 1 and strain 2 against Silver Nanoparticles (SNPs) of *Azadirachta indica* (Neem) was 6±1.41, 5.5±1.56. Zone of inhibition ± S.E value of strain 1

and strain 2 against SNPs of *Calotopis procera* was 8.25±1.37, 7.5 ±0.87. Zone of inhibition ± S.E value of strain 1 and strain 2 against SNPs of *Cassia fistula* was 8±1.78, 5.5±1.44mm. Zone of inhibition ± S.E value of strain 1 and strain 2 against SNPs of *Eucalyptus radiata* was 6.25±0.48, 3.25±0.48 .

One way ANNOVA test applied to silver nanoparticles of *Azadirachta indica*, *Calotropis procera*, *Cassia fistula* and *Eucalyptus radiata*. All silver nanoparticles synthesized from different green plant extracts showed similar antibacterial activity because p value was greater than 0.05. Tuckey test and Bonferroni test showed that p value was greater than 0.05 for all the comparisons so there was no statistically significant difference between them (Figures 7, 8, 9).

Antibacterial activity test of Plant extracts

Plant extracts of *Azadirachta indica* (Neem), *Calotropis procera*, *Cassia fistula* and *Eucalyptus radiata* showed no zone of inhibition around all bacterial strains. All isolated bacterial strains showed resistance against all plant extracts (Figure 10).

Accession number of isolated strains

According to molecular characterization by 16SrRNA sequencing results Strain 1 showed similarity with *Escherichia coli (E.coli)* PQ549958 and strain 2 showed similarity with *Enterococcus* species PQ561524.

Table 1. Streaking type of isolated bacterial strains

Isolated Strains	**Streaking Type**
Strain1	Continuous streaking
Strain 2	Quadrant streaking

Table 2. Blood agar test of isolated bacterial strains

Strains	**Pathogenicity**	**Hemolysis**
Strain1	+ve	Beta Hemolysis
Strain 2	+ve	Beta Hemolysis

Table 3. Antibiotic resistance of isolated bacterial strains ± S.E value (Disc diffusion method)

Strains	**Zone of inhibition (mm)**	
	Meronem	**Cefixime**
Strain 1	14±0	Resistant
Strain 2	10.25±0.63	2.75±0.25

R= Resistance of isolated bacterial strain against antibiotic.

Table 4. Antibiotic resistance of isolated bacterial strains ± S.E value (Well diffusion method)

	Zone of inhibition (mm)

	Strain 1	**Strain 2**
Doxycycline (DOX)	12.25±0.5	12.25±6.08
Oxytetracyline (TET)	9.25±0.5	4.75±0.5
Ciprofloxacin (CIP)	9.75±0.5	7.5±0.5
Chloramphenicol (CHL)	5±0	7.5±1
Ceftriaxone (CEF)	12.75±2.06	Resistant
Amoxicilin (AMO)	11.25±0.96	6.75±2.06
Azomax (AZO)	11.25±0.96	10.75±0.5

R= Resistance of isolated bacterial strain against antibiotic

Table 5. Antibacterial activity test of Nanoparticles

	Zone of inhibition (mm)			
Strains	**SNPs of *Azadirachta indica***	**SNPs of *Calatropis procera***	**SNPs of *Cassia fistula***	**SNPs of *Eucalyptus***
Strain 1	6±1.42	8.25 ± 1.38	8± 1.78	6.25 ±0.48
Strain 2	5.5±1.56	7.5 ±0.87	5.5± 1.45	3.25 ± 0.48

Table 6. Antibacterial activity test of Plant Extracts

Strains	**Zone of inhibition(mm) Zone of inhibition(mm)**			
Strains	**SNPs of *Azadirachta indica***	**SNPs of *Calatropis procera***	**SNPs of *Cassia fistula***	**SNPs of *Eucalyptus***
Strain 1	R	R	R	R
Strain 2	R	R	R	R

Figure 1. Spreading on plates of collected samples

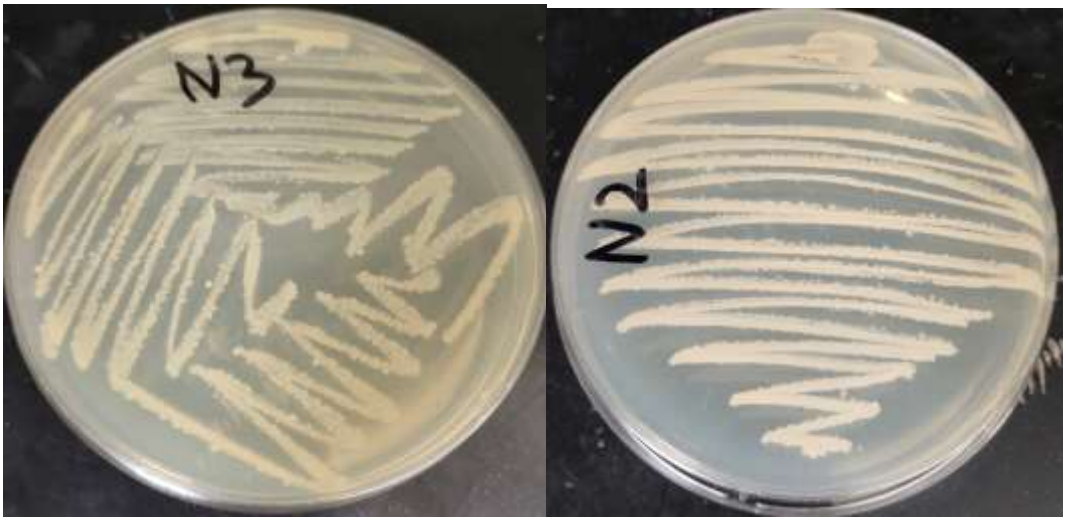


Figure 2. Streaking plates of isolated colonies

Figure 3. Blood agar test of isolated bacterial strains

Strain 1 **Strain 2**

Figure 4. Antibiotic resistance of isolated bacterial strains

Disc diffusion method, cefixime and meronem discs were used to check the antibacterial activity of isolated strains.

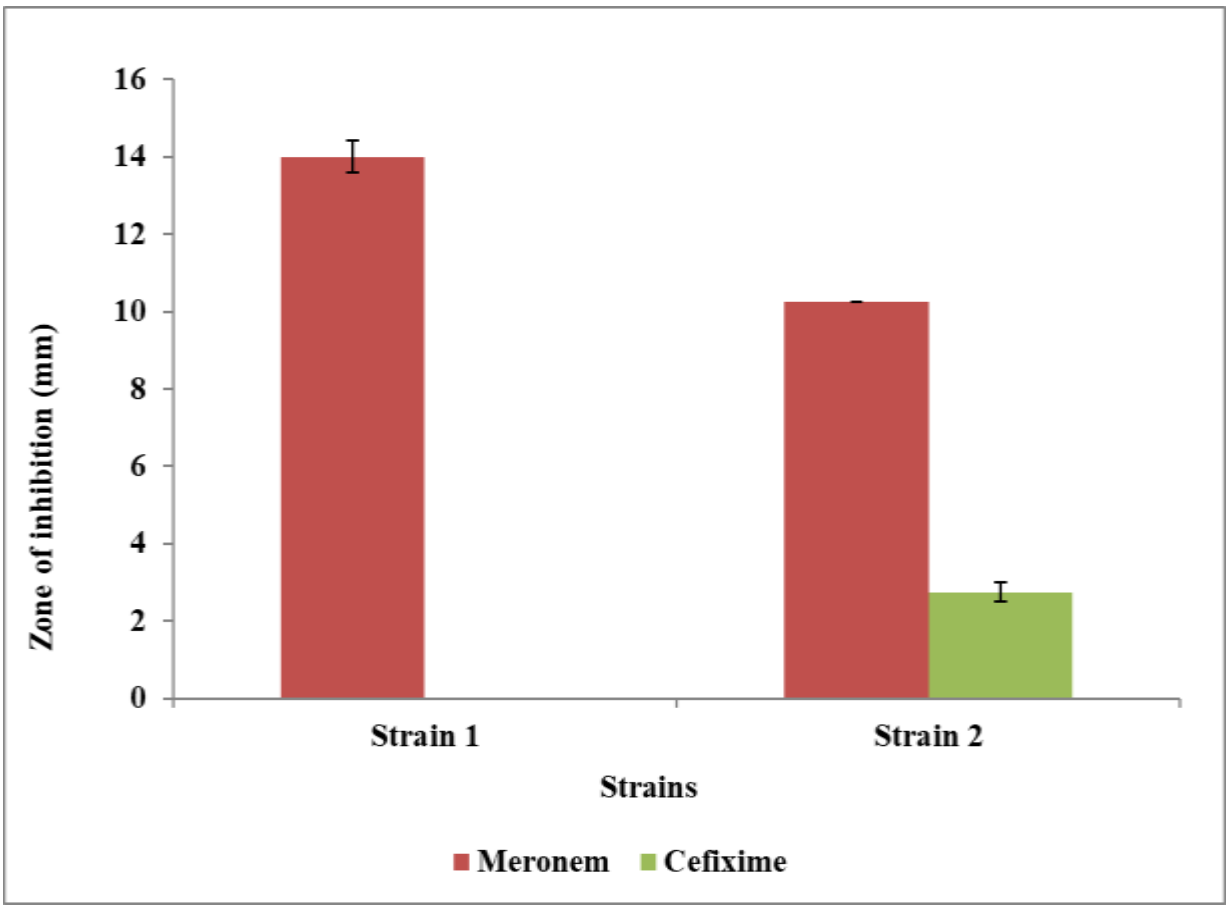
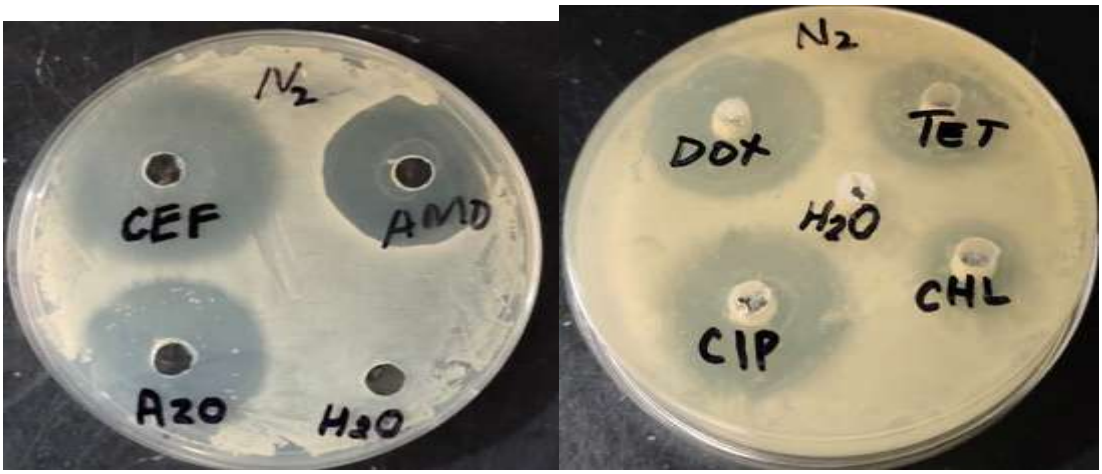


Figure 5. Graph shows antibiotic resistance of isolated bacterial strains



Strain 1 **Strain 1**

Strain 2 **Strain 2**

Figure 6. Antibiotic resistance of isolated bacterial strains

In this figure DOX, TET, CIP, CHL, CEF, AMO and AZO is an abbreviated form of Doxycycline, Oxytetracycline, Ciprofloxacin and Chloramphenicol, Ceftriaxone, Amoxicillin and Azomax.

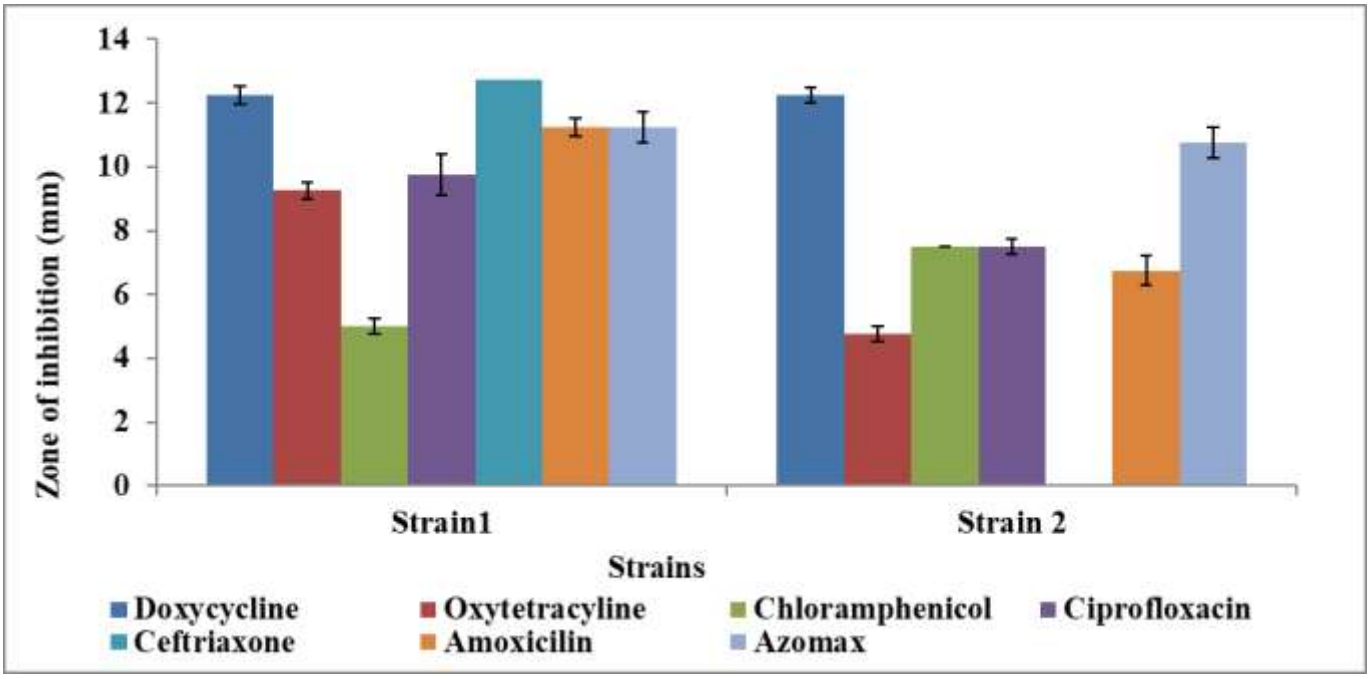
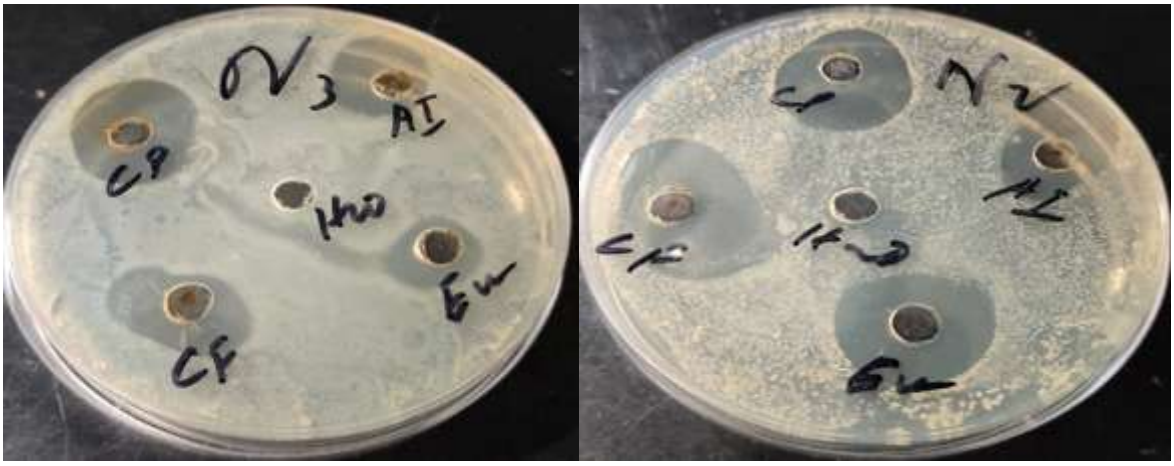


Figure 7. Graph shows antibiotic resistance of isolated bacterial strains



Strain 1 **Strain 2**

Figure 8. Antibacterial activity test of Nanoparticles

AI , CP, CF and EU is an abbreviated form of *Azadirachta indica* (neem) , *Calotropis procera*, *Cassia fistula* and *Eucalyptus radiata*

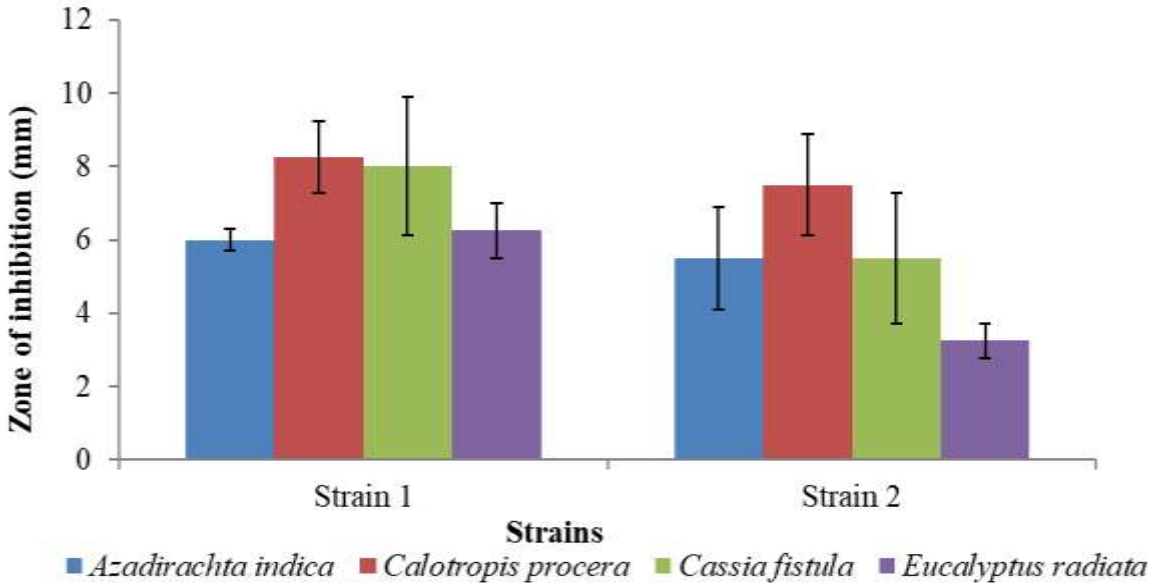
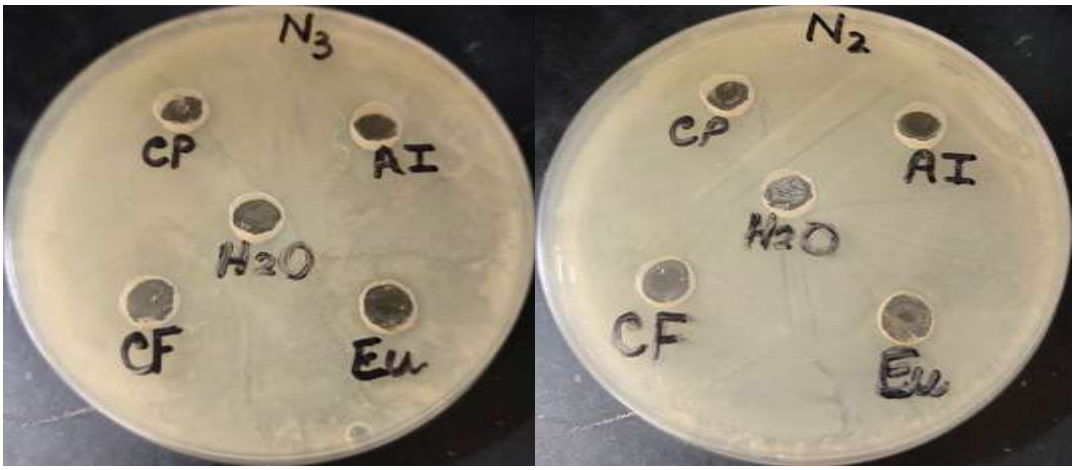


Figure 9. Graph shows antibacterial activity of nanoparticles against isolated bacterial strains



Strain 1 **Strain 2**

Figure 10. Antibacterial activity test of Plant extracts

AI , CP, CF and EU is an abbreviated form of *Azadirachta indica* (neem) , *Calotropis procera*, *Cassia fistula* and *Eucalyptus radiate*

Partial sequence of Strain 1(*E.coli*) (PQ549958)

AGTGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCAT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATC
CACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTA
CGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCA
TTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC
CTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAAC
AAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCT
GACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGCACCTTTCCATCTCTGA
AAAGTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACC
ACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGC
CGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGC
ACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTT
GCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCA
CCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTC
TACGAGACTCAAGCTTGCCAGTATCAGATGCAGTTCCCAGGTTGAGCCCGGGGATTT
CACATCTGACTTAACAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACG
CTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGC
GGGTAACGTCAATGAGCAAAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTAC
TTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCA
TTGTGCAAAATTCCCCCTGCTGCCTCCCGTAGAATCTGGACCGGGTCAGTTCCGGGTG
GCTGGCCTCCTCTCAGACAGCTAGGGATCGTCGCCTTGGTGAGCCTTACCCACCAAA
AGCTATCCATTGGGCCATCCATGTGAAAAGGCCCAAGGCCCCCTTTT

Partial sequence of Strain 2 (*Enterococcus* sp.) (PQ561524)

CGTTCCCTTAGGCGGCTGGCTCCAAAAAGGTTACCCCACCGACTTCGGGTGTTACAA
ACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCG
TGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAAT
CCGAACTGAGAAAAGTTTTAAGAGATTTGCTTGACCTCGCGGTCTAGCGACTCTTTGT
ACTTCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC
ATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAACTAAATGA
TGGCAACTAACATTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC
ACGAGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCGAAGGAAAAGCCCTA
TCTCTAGGGTGGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAAT
TAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCT
TGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTGAAGGGCG
GAAACCCTCCAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAAT
CCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCC
TTCGCCACTGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCAC
TCTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGGG
GGCTTTCACATCAGACTTAAGAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGG
ACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTT
CTGGTTAGATACCGTCAGGGG

1. Discussion

The result of current study showed that no resistance shown by any isolated bacterial strain against meronem. According to this study results strain 1 showed more susceptibility and maximum zone of inhibition was 14 mm against meronem. Strain 2 showed less susceptibility and minimum zone of inhibition was 10.25 mm against meronem. Results from different previous studies showed that meronem was considered as good antibacterial agent because of its efficacy and showed less resistance against different gram positive and gram negative bacterial species like *Pseudomonas* species (Chaudhari *et al*., 2013).

The result of current study showed resistance of strain 1 against cefixime and strain 2 also showed minimum zone of inhibition around 2.75 mm. Different past studies showed higher resistance of different bacterial strains against cefixime (Ayatollahi *et al*., 2013).In previous studies high resistance rate against cefixime was observed in *Pseudomonas* and *Klebsiella* spp. In many past findings highest ratio of resistant bacteria against cefixime also observed in different hospital effluents and surface water rivers due to overuse of antibiotic (Chowdhury and Uddin, 2022).

In the current study doxycycline antibiotic showed that maximum zone of inhibition was 12.25 mm against strain 1 and strain 2. No resistance shown by any strain against doxycycline. In the previous studies results showed that doxycycline was used against multi drug resistant bacteria like *E.coli* and *P.aeruginosa* due to its efficacy and less susceptibility to different pathogenic bacteria (Lai *et al*., 2016). In the current study oxytetracycline antibiotic showed that maximum zone of inhibition was 9.25 mm against strain 1 and zone of inhibition measured around strain 2 was 4.75mm. Previous studies results showed that different pathogenic bacteria gain resistance against tetracyclines and oxytetracyclines (Grossman, 2016). In the current study ceftriaxone showed resistance against strain 2 and measured zone of inhibition around strain 1 was 12.75mm. Results from past studies showed resistance of ceftriaxone against some gram positive and negative bacteria like *Staphylococcus aureus*, *Pseudomonas aeroginosa* and *E. coli* (Gelaw *et al*., 2022).

In this study strain 1 showed more susceptibility to ciprofloxacin and zone of inhibition was 9.75mm and zone of inhibition around strain 2 was 7.5mm. In the past studies results showed that ciprofloxacin used against different gram positive and negative bacteria due to its efficacy and susceptibility to different pathogenic bacteria (Rodriguez *et al*., 2015). In this study strain 2 showed more susceptibility to chloramphenicol and zone of inhibition was 7.5 and less susceptibility shown by strain 1 and zone of inhibition was 5. Past studies results showed that chloramphenicol used against *E.coli* and *S.aureus* bacteria and showed resistance in some strains of *E. coli* and *Pseudomonas* spp. (Carone *et al*., 2014). In this study strain 1 showed more susceptibility to amoxicillin and zone of inhibition was 11.25mm and less susceptibility shown by strain 2 and zone of inhibition was 6.75mm. Previous studies results showed that amoxicillin used against *Streptococcus* and *Enterococcus* spp. and showed resistance in some *Klebsielle* and *P.aeruginosa* spp. (Mulu *et al*., 2017). In this study strain 1 showed more susceptibility to azomax and zone of inhibition was 11.25mm and less susceptibility shown by strain 2 and zone of inhibition was 10.75mm. Previous studies results showed that azomax used against multi drug resistant bacteria like *E.coli* (Meerwein *et al*., 2020).

The results of current study showed that silver nanoparticles synthesized from different green plants including silver nanoparticles of *Azadirachta indica*, *Cassia fistula*, *Calotropis procera* and *Eucalyptus radiata* showed antibacterial activity. The result of current study showed that maximum zone of inhibition was 6mm against strain 2 and no resistance was shown by any

strain against silver nanoparticles of *Azadirachta indica*. Previous studies result showed that silver nanoparticles of *Azadirachta indica* showed good antibacterial activity against different gram positive bacteria like *S.aureus* and *B.subtilis* and gram negative bacteria like *E.coli* and *P. aeruginosa* (Ahmed *et al*., 2016 and Bhat *et al*., 2019).

The result of this study showed that maximum zone of inhibition was 8.25mm against strain 1 and no resistance was shown by any strain against silver nanoparticles of *Calotropis procera.* In past studies it was observed that silver nanoparticles of *Calotropis procera* showed maximum antibacterial activity against gram negative bacteria like *E.coli* and *P.aeruginosa* (Mohamed *et al*., 2014 and Sagadevan *et al*., 2020). The result of current study showed that maximum zone of inhibition was 8 mm against strain 1 and no resistance was shown by any strain against silver nanoparticles of *Cassia fistula*. In the past studies it was observed that silver nanoparticles of *Cassia fistula* showed maximum antibacterial activity with 95 percent inhibitory effect against gram positive bacteria including *B.subtilis* and *S.aureus* and showed susceptibility and minimum antibacterial activity against gram negative *E.coli* and *P.aeruginosa* (Mohanta *et al*., 2016). The result of current study showed that maximum zone of inhibition was 6.25 mm against strain 1 and no resistance was shown by any strain against silver nanoparticles of *Eucalyptus* spp. In past studies SNPs of *Eucalyptus* showed good antibacterial activity against some gram positive bacteria including *S.aureus* or *B.subtilis* and gram negative bacteria including *E.coli* and *P.aeruginosa* (Mohammed, 2015).

In current study all isolated bacterial strains showed resistance to all four aqueous plant extracts including *Azadirachta indica*, *Cassia fistula*, *Calotropis procera* and *Eucalyptus radiata*. In past studies methanolic and water extracts of *Azadirachta indica* was used to assess the antibacterial activity of *E.coli* and *Staphylococcus aureus* and results showed that aqueous extracts of *Azadirachta indica* did not show any good antibacterial activity against different pathogenic bacteria like *Staphylococcus aureus* and *E.coli*. While methanolic extracts show good antibacterial activity at different concentrations (Francine *et al*., 2015 and Rathod *et al*., 2012).

In past studies *Cassia fistula* water plant extracts showed antibacterial activity against gram positive bacteria in comparison with methanolic extracts of *Cassia fistula* that showed good antibacterial activity against different gram positive and gram negative bacteria (Panda *et al*., 2011). In past studies *Calotropis procera* water plant extracts showed good antibacterial activity against gram positive and gram negative bacteria (Mainasara *et al*., 2012 and Yesmin *et al*., 2008). In previous studies Eucalyptus spp. water plant extracts showed good antibacterial activity against gram positive bacteria but showed resistance to different gram negative bacteria (Bencheikh *et al*., 2021).

According to molecular characterization by 16SrRNA sequencing results Strain 1 showed similarity with *Escherichia coli (E.coli)* and strain 2 showed similarity with *Enterococcus* species.

The study concluded that due to different anthropogenic activities river Ravi water does not suitable for irrigation purpose for the cultivation of crops and these crops as raw vegetables consumed by humans and ultimately cause different severe gastrointestinal infections in humans due to presence of pathogenic bacteria in water. Due to overuse and misuse of antibiotics used to treat bacterial infections, bacteria developed resistance against different antibiotics. It is suggested that different biological tools like nanoparticles and plant extracts should be tested more as antibacterial tools against which pathogens might not developed resistance.

Ethical Approval

Ethical approval was not required for this article

Consent to Participate
Not required
Consent to Publish
All authors gave permission for publication in this journal.
Authors' contribution
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Methodology: Nida Mazhar, Maham Chaudhry
Figures and Graphs: Memuna Ghafoor Shahid, Faiqa barkat, Marriam Wasif
Funding
None
Competing Interests
No competing interests among authors.
Data Availability statement
Data is available at NCBI website. Also the Accession numbers are provided in Manuscript.
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