Acta Scientific Microbiology (ISSN: 2581-3226)

Volume 9 Issue 7 July 2026

Research Article

Elucidating the Antibacterial and Antibiofilm Efficacy of *Carum copticum* Against Multidrug-Resistant Bacteria of Wastewater Origin

Rahisuddin Khan, Shirjeel Ahmad Siddiqui, Vishnu AL and Iqbal Ahmad*

Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, Uttar Pradesh, India

***Corresponding Author:** Iqbal Ahmad, Professor, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, 202002, UP, India.

DOI: 10.31080/ASMI.2026.09.1612

Received: May 19, 2026

Published: July 03, 2026

Abstract

Antimicrobial resistance (AMR) is a major global health concern, and wastewater environments serve as important reservoirs for the dissemination of multidrug-resistant (MDR) bacteria. In this study, 70 Gram-negative bacterial isolates were obtained from four wastewater sites in Aligarh and tentatively identified as *Escherichia coli*, *Enterobacter* spp., *Citrobacter* spp., *Proteus* spp., *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., and *Serratia* spp. Antibiotic susceptibility testing revealed the highest resistance to ampicillin and amoxicillin (100%), rifampicin (82%), erythromycin (73%), and nalidixic acid (58%), while lower resistance was observed against chloramphenicol (18%), nitrofurantoin (10%), and aminoglycosides (1.5-7%). β-lactamase production was detected in 24 isolates, and 10 were confirmed as ESBL producers by PDCT. Biofilm analysis showed strong biofilm formation in 32.48% of isolates. The antibacterial and antibiofilm activities of nine medicinal plant extracts were evaluated against strong biofilm-forming strains and standard cultures (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* MTCC 737, and *Pseudomonas aeruginosa* PAO1). Among the nine medicinal extracts, *Carum copticum* and *Terminalia chebula* exhibited activity against all standard cultures and *E. coli* isolates. Based on initial screening, *Carum copticum* was selected for detailed study against a strong biofilm-forming *E. coli* isolate (SAS5). Showing a dose-dependent reduction in biofilm formation, with maximum inhibition observed at MIC/2 concentration (69.3%) of *Carum copticum*. Scanning Electron Microscopy analysis revealed significant biofilm inhibition by *Carum copticum* extract on the SAS5 strain compared to the control. The study highlights wastewater as a significant source of MDR bacteria and demonstrates the potential of medicinal plant extracts as alternative antibacterial and antibiofilm agents against drug-resistant pathogens.

Keywords: Antimicrobial Resistance; ESBL; Plant Extracts and Antibiofilm Agent

Introduction

Antimicrobial resistance (AMR) is recognized as a major global public health challenge that threatens the effective treatment of infectious diseases worldwide, according to the World Health Organization [1]. Wastewater ecosystems are widely recognized as significant reservoirs for the persistence and spread of multidrug-resistant (MDR) bacteria and antibiotic resistance genes [2]. Various types of wastewaters, including hospital, municipal, and household wastewater, contain antibiotic residues, disinfectants, heavy metals, and harmful microbes. These substances exert selective pressure, which in turn encourages the development of resistant bacterial populations [3]. Gram-negative bacteria found in the environment, such as *Escherichia coli, Klebsiella* spp., *Pseudomonas* spp., and *Enterobacter* spp., are frequently collected from wastewater systems. These bacteria are known to carry multiple antibiotic resistance determinants [4]. These adaptable bacteria can spread throughout aquatic ecosystems and ultimately infect humans through polluted water and other food chains. The production of β-lactamases, including extended-spectrum β-lactamases (ESBLs), is a significant resistance mechanism in environmental bacteria. This mechanism confers resistance to broad-spectrum β-lactam antibiotics [5]. Research indicates that ESBL-producing Enterobacteriaceae are more prevalent in wastewater settings [6]. Furthermore, bacteria that are resistant to multiple medications often exhibit a strong capacity to form biofilms, which not only helps them survive harsh environmental conditions but also increases their resistance to antimicrobial drugs [7]. Biofilms are organized communities of microorganisms encased in extracellular polymeric substances. These compounds protect bacterial cells against the effects of some medications and environmental stresses. According to Ahmad., *et al*. [8], wastewater pipes and treatment facilities create environments favorable to biofilm formation and the horizontal transmission of resistance genes. The persistent presence of bacteria resistant to many drugs and associated with biofilms in wastewater is a serious problem for both the environment and human health [9]. In view of the growing problem of AMR, medicinal plants are being extensively researched as potential alternative sources of antibacterial and antibiofilm agents. It has been demonstrated that phytochemicals derived from plants, such as phenolics, alkaloids, flavonoids, and terpenoids, possess a wide range of antibacterial characteristics [10]. As reported in studies [11], *Carum copticum* has been the subject of significant research due to its antibacterial properties, attributed to thymol and other associated bioactive compounds.

This study focuses on the isolation and characterization of multidrug-resistant Gram-negative bacteria from wastewater, the assessment of their antibiotic resistance, β-lactamase and ESBL production, and biofilm-forming ability, and the evaluation of the antibacterial and antibiofilm potential of selected medicinal plant extracts, including *Carum copticum*.

Materials and Methods

Sample collection and isolation of Gram-negative bacteria

Wastewater samples were collected mainly from four sites in Aligarh city, namely Jamalpur sewage drain, Habib Hall canteen outlet, University sewage pumping station, and JNMC trauma center sewage drain, during December-April 2018 and subsequently repeated for the same duration in 2019. Sampling locations were carefully selected based on hospital discharge points, population density, and environmental significance to ensure comprehensive representation of potential contamination sources. Samples were collected in sterile glass bottles and transported to the laboratory immediately for initial processing, in accordance with APHA guidelines. After bringing the samples to the laboratory, sample was appropriately diluted in sterile NSS. For each dilution, 0.1 ml of the sample was spread onto EMB (Eosin Methylene Blue) and MacConkey agar media, and incubated at 37 °C for 18–24 h. Distinct colonies were purified, characterized, and stored on nutrient agar slants at 4 °C, with long-term preservation in 50% (v/v) glycerol stocks at−80 °C.

Polyphasic characterization of isolates

The bacterial colonies were examined for distinct morphological characteristics, including shape, size, and color. The isolates were further analyzed by Gram staining and microscopic examination. Biochemical tests were performed to tentatively identify the bacteria and to differentiate the members of the Enterobacteriaceae family, in accordance with established protocols [12]. The phylogenetic position of the strain SAS5 was determined using the method described by [13], based on partial 16S rRNA gene sequences and comparisons with available sequences in the DNA databank. A neighbor-joining phylogenetic tree was constructed from the partial 16S rRNA gene sequence, and the tree topology was validated using bootstrap analysis with 1000 resamplings.

Antimicrobial susceptibility testing

Antimicrobial Susceptibility Testing (AST) The isolated Gram-negative bacteria were assessed by the disc diffusion assay on Mueller–Hinton agar (MHA) plates, following the standard guidelines (Bauer., *et al*. 1966). One hundred μL of each bacterial suspension was uniformly spread over the Mueller-Hinton Agar (MHA) plates. Antibiotic discs with specific potencies (μg/disk) were taken such as, ampicillin (10), gentamicin (10), tetracycline (30), erythromycin (15), rifampicin (5), azithromycin (10), nalidixic acid (30), nitrofurantoin (30), chloramphenicol (30), Norfloxacin (10), lomefloxacin (30), amoxicillin (30), co-trimoxazole (30), kanamycin (30) and streptomycin (10) (HiMedia) were used. Each disc was placed on the agar surface with sterile forceps, then incubated at 37 °C for 18 h. Inhibition zones around the discs were measured in mm using a zone-measuring scale, and the results were interpreted according to CLSI guidelines [14].

Minimum Inhibitory Concentration (MIC) of selected antibiotics against selected MDR isolates

Broth microdilution was used to calculate MIC values per CLSI guidelines [15]. Luria-Bertani broth (HiMedia) was added to each well, followed by 100 µL of the test isolate. Each antibiotic (chloramphenicol, ampicillin, tetracycline, norfloxacin, azithromycin, cefotaxime, and nalidixic acid) was added to the 96-well microtiter plate at varying concentrations ranging from 2 µg/mL to 1024 µg/mL. The suspected wells had low turbidity, and inoculating 0.1 ml of broth onto nutrient agar plates indicated MIC by the absence of growth.

Assay for β-lactamase production

β-lactamase production in the strains was detected using the rapid iodometric method [16,17]. The test isolates were grown overnight on a nutrient agar plate amended with ampicillin and penicillin (25 µg/mL each). The culture was scraped and transferred to sugar tubes having ampicillin and penicillin solution (6000 µg/ml). After 30 minutes at room temperature, 2 drops of starch solution (1% (w/v) in distilled water) and a drop of iodine reagent (2% (w/v) iodine in 53% (w/v) aqueous potassium iodide) were added to the reaction mixture, leading to the development of blue color due to the reaction of the iodine with the starch. The sugar tube was shaken for 1 min at room temperature. Rapid decolorization of blue color indicated the presence of β-lactamase enzyme. Persistence of the blue color for longer than 10 min constituted a negative test and indicated that the penicillin and ampicillin molecules had not undergone β-lactam ring cleavage.

Screening for Extended-Spectrum β-Lactamase (ESBL) production in test isolates

ESBL production was detected using the Phenotypic Disc Confirmation Test (PDCT) according to CLSI guidelines [15]. The isolates showing beta-lactamase activity were selected for ESBL testing. For this test, bacterial cultures were grown overnight in nutrient broth and evenly spread on Mueller–Hinton agar (MHA) plates. Antibiotic discs of cefotaxime (30 µg) and ceftazidime (30 µg), alone and in combination with clavulanic acid (30/10 µg), were placed on the agar surface. The plates were incubated at 37 °C for 18-24 h. ESBL production was confirmed by an increase of ≥5 mm in the inhibition zone diameter around the antibiotic disc combined with clavulanic acid compared to the antibiotic disc alone. Positive and negative control strains were included in the test.

Screening of isolates for Biofilm formation

Biofilm formation was measured using the polystyrene microtiter plate assay [18]. Briefly, overnight cultures of the isolated strains were resuspended in fresh Luria-Bertani (LB) broth and incubated in polystyrene microtiter plates at 30 °C for 24 h. Following incubation, the plates were rinsed to remove planktonic cells, and the adhered biofilms were stained with 0.1% w/v crystal violet solution. The surface-attached cells were quantified by solubilizing the bound dye with 95% ethanol, and absorbance was measured at 600 nm.

Collection of plant extracts

A total of nine methanolic plant extract stock solutions were obtained from the Department of Agricultural Microbiology, Faculty of Agricultural Sciences, AMU, Aligarh. The extract stock solutions were reconstituted in 1% DMSO to obtain the desired concentrations.

S. no	Plant extracts	Stock concentration (mg/ml)	Part used	Uses	References
1	*Cyperus scariosus*	403	Leaves	Used in treating nausea, fever, and inflammation.	Babiaka., *et al*. 2025
2	*Syzygium cumini*	560	Seeds	Used for controlling diabetes and for aesthetic purposes.	Kumar., *et al*. 2025

3	*Hemidesmus indicus*	670	Roots	Decoction or syrup is used as a refreshment syrup and as medicine.	Manjulatha., *et al*. 2014
4	*Holorrhena anti-dysentrica*	610	Bark	Useful in treating gut motility disorders.	Tiwari., *et al*. 2024
5	*Terminalia chebula*	580	Fruit	Effective in treating Alzheimer’s.	Gao., *et al*. 2024
6	*Carum carvi*	690	Fruit	Used as spices, as a fragrance component, and as a breath freshener.	Agnihotri., *et al*. 2024
7	*Embelia ribes*	600	Seeds	Used as an anti-helminthic and carminative.	Sharma., *et al*. 2022
8	*Commiphora wightii*	590	Resins	Used as a fragrance component and to maintain blood cholesterol levels.	Sarup., *et al*. 2022
9	*Carum copticum*	529	Seeds	Effective in treating stomach disorders and is also used as a carminative.	Akbar., *et al*. 2020

Table a

Bioassay for anti-bacterial and anti-biofilm activity

Antibacterial activities of the plant extracts against strong biofilm-forming isolated strains and standard strains (*E. coli* ATCC 25922, *Staphylococcus aureus* MTCC 737, and *Pseudomonas aeruginosa* PAO1) using the agar well diffusion method [19]. Mueller–Hinton agar plates containing 30 mL of medium were inoculated with freshly grown bacterial cultures. Wells were aseptically prepared using a sterile cork borer, sealed with soft agar, and loaded with 50 µL of plant extracts at the varying concentrations. Following incubation at 37 °C for 24 h, the inhibition zones were measured and recorded. The effect of sub-MIC concentrations of *Carum copticum* on a strong biofilm-forming *E. coli* strain SAS5 was evaluated using the polystyrene microtiter plate assay [18]. Briefly, 1% overnight cultures of biofilm-forming isolates were inoculated into fresh LB medium with or without sub-MIC levels of the extracts and incubated at 37 °C for 24 h. Planktonic cells were removed using sterile PBS, and adherent cells were washed, air-dried, and stained with 0.1% w/v crystal violet for a duration of 10-15 minutes, followed by rinsing with sterile PBS to eliminate unbound crystal violet. The bound dye was solubilized in 95% ethanol, and absorbance was measured at 600 nm. Biofilm inhibition was calculated as the percentage reduction in OD compared to untreated controls.

The percentage inhibition of biofilm was calculated utilizing the following formula.

(% Percentage of inhibition = Control OD_{600nm} – Test OD_{600nm}/ Control OD_{600nm} × 100)

The biofilm architecture of the SAS5 strain, treated and untreated, was examined using scanning electron microscopy (SEM). For SEM, bacterial isolates were grown on sterile glass coverslips in LB broth at 37 °C for 24 h. Non-adherent cells were removed by washing with PBS (pH 7.2), followed by fixation in 2.5% glutaraldehyde for 6 h. Samples were dehydrated through a graded ethanol series (30–100%), air-dried, gold-coated, and visualized using a scanning electron microscope (JEOL JSM-6510 LV) at USIF, AMU, Aligarh.

Statistical analyses

All experiments were performed in triplicate, and the data were presented as the mean or mean ± standard deviation (SD), as applicable.

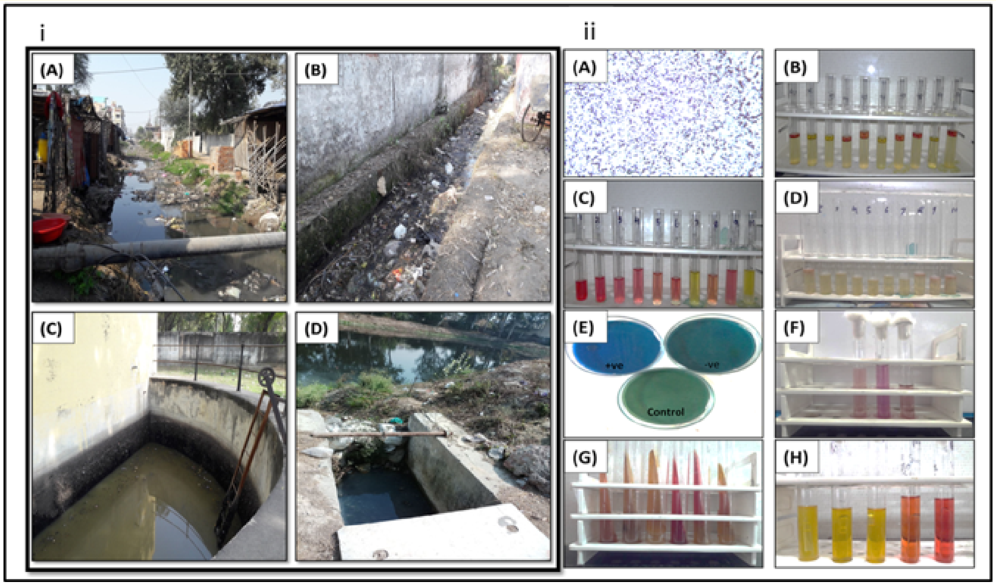
Results

Isolation of Gram-negative bacteria and their identification

A total of 70 bacterial isolates were isolated from 4 different waste water sites of Aligarh city (U.P) i.e., Jamalpur sewage drain, Habib hall canteen outlet, University sewage pumping station and JNMC Trauma Centre sewage (Supplementary table 1, Supplementary figure 1(i)) and all are tentatively identified as *Enterobacter sp., E.coli, Citobacter sp., Proteus sp., Pseudomonas* *sp., Salmonella sp., Shigella sp., Klebsiella sp. and Serratia sp.*, based on the morphological, cultural and biochemical characteristics in accordance with the Bergey's Manual of Determinative Bacteriology. Gram's staining was performed, and all of them were found to be Gram-negative short rods. The isolates were further characterized biochemically. About 50% of the test isolates after screening were positive for Indole, 58% were positive for methyl red, 28% for Voges-Proskauer, and 75% positive for Simmons' citrate test (Supplementary Table 2, Supplementary Figure 2). About 28% of the isolates were positive for Urease production. 20% strains showed a positive reaction for H_2S production. Distinct biochemical reaction patterns were observed among Enterobacter, Pseudomonas, Citrobacter, Salmonella, and Shigella spp., facilitating their preliminary identification (Supplementary Table 2). The 16S rRNA gene sequence obtained in this study was submitted to GenBank under accession number PP808676.1. BLAST analysis revealed that strain SAS5 showed the highest similarity (99.6%) with *Escherichia coli* strain S3-4-1. A neighbor-joining phylogenetic tree based on the partial 16S rRNA gene sequence and its closest phylogenetic neighbors is presented in Figure 1.

S. No.	Wastewater samples	No. of samples collected	Percentage of bacterial isolates obtained
1	Jamalpur main sewage drain	5	44
2	Habib hall canteen outlet	5	11
3	University sewage pump, Safi Road	5	21
4	Trauma centre, JNMC	5	23

Supplementary Table 1: Sample collection sites and percentage distribution of bacterial isolates obtained from different sampling locations.



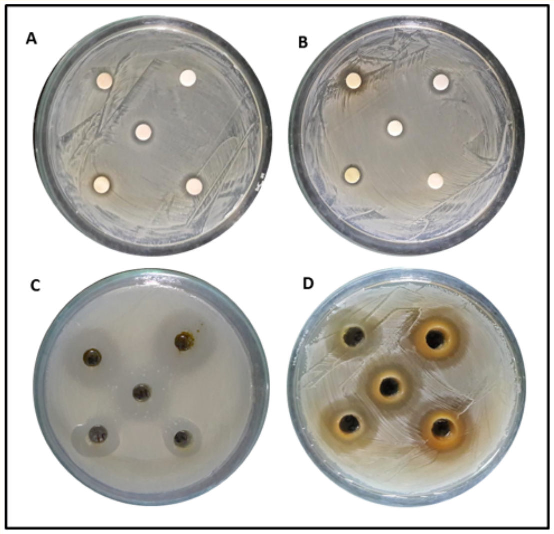
Supplementary Figure 1: Wastewater sampling sites and biochemical characterization of bacterial isolates. In 1(i), Panel A shows the Jamalpur sewage drain; Panel B shows the Habib Hall canteen outlet; Panel C shows the University sewage pumping station; and Panel D shows the JNMC Trauma Center sewage drain. In 1(ii), Panel A shows Gram-negative rod-shaped cells, Panel B shows the indole test, Panel C shows the methyl-red test, Panel D shows the Voges-Proskauer test, Panel E shows the citrate utilization test, Panel F shows the urease test, Panel G shows triple sugar iron (TSI) agar slants, and Panel H shows the sugar fermentation test.

Citation: Iqbal Ahmad*., et al.* "Elucidating the Antibacterial and Antibiofilm Efficacy of *Carum copticum* Against Multidrug-Resistant Bacteria of Wastewater Origin". *Acta Scientific Microbiology* 9.7 (2026): 29-43.

Gram staining	Colony morphology on SS agar	Colony morphology on EMB Agar	I	M.R	V.P	O	C	U	TSI	Gas production	H_2S	G/G	F/G	S/G	M/G	L/G	Tentative identification	Total number of isolates (%)
-	Slightly pink	Green sheen	+	+	-	+	-	-	A/A	+	-	+/+	+/-	+/+	-/-	+/-	*E. coli*	8.57
-	Colorless with black centers	Colourless	-	+	-	+	+	-	K/A	-	+	+/+	+/-	-/-	+/-	-/-	*Salmonella sp.*	11.43
-	Colourless	Colorless	-	-	-	+	+	-	K/K	-	-	-/-	-/-	-/-	-/-	-/-	*Pseudomonas sp.*	17.14
-	Red	Pink mucoid	+	-	+	+	+	+	A/A	+	-	+/+	+/+	+/-	+/+	+/+	*Klebsiella sp.*	7.14
-	Colorless	Colorless	+	+	-	+	-	-	K/A	-	-	-/-	+/-	-/-	+/+	-/-	*Shigella sp.*	1.43
-	Colorless	Colorless	-	+	+	-	+	-	K/A	-	-	+/+	+/+	+/+	+/+	-/-	*Serratia sp.*	2.86
-	Colorless with black centers	Colorless	-	+	-	+	+	+	K/A	+	+	+/+	+/+	-/-	-/-	-/-	*Proteus sp.*	8.57
-	Red	Pale sheen	+	+	-	+	+	-	A/A	+	-	+/+	-/-	+/-	+/+	+/-	*Citrobacter sp.*	27.14
-	Pale	Pink mucoid	-	-	+	-	+	-	A/A	+	-	+/+	+/+	+/+	+/+	+/+	*Enterobacter sp.*	15.72

SS: Salmonella- Shigella agar; EMB: Eosin Methylene Blue agar; I: Indole test; MR: Methyl Red; VP: Voges–Proskauer; O: Oxidase test; C: Catalase test, U: Urease Test; TSI: Triple Sugar Iron; A/A = acidic slant/acidic butt; K/A = alkaline slant/acidic butt; K/K = alkaline slant/alkaline butt. H2S: Hydrogen Sulfide, G/G: Glucose/Gas; F/G: Fructose/Gas; S/G: Sucrose/Gas; M/G: Maltose/Gas; L/G: Lactose/Gas, (+) indicates positive biochemical reaction/sugar fermentation; (-) indicates negative biochemical reaction/sugar fermentation

Supplementary Table 2: Morphological and Biochemical characteristics of isolated Gram-negative bacteria.



Supplementary Figure 2: Antibacterial activity of *Carum copticum* and *Terminalia chebula* against E. coli isolate determined by disc diffusion and well diffusion methods. Panels A and B show the antibacterial activity of *Carum copticum* and *Terminalia chebula*, respectively, using the disc diffusion method, whereas Panels C and D show their antibacterial activity using the well diffusion method.

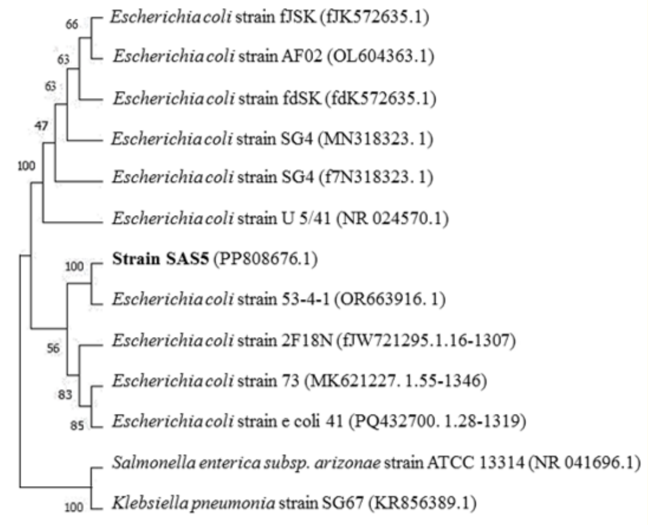
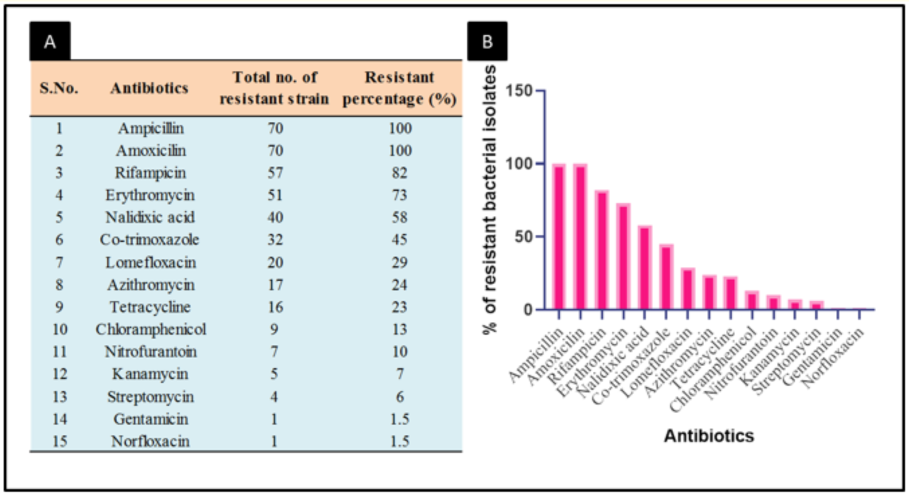


Figure 1: A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic position of the strain SAS5 isolated from wastewater.

Assessment of antibiotic resistance in isolated Gram-negative bacteria

The antibiotic sensitivity of the test isolates was evaluated against 15 antibiotics using the disc diffusion method (Figure 2A). Based on the sensitivity patterns, the incidence of resistance was determined. All isolates exhibited 100% resistance to ampicillin and amoxycillin, followed by rifampicin (82%), erythromycin (73%), nalidixic acid (58%), co-trimoxazole (45%), and lower resistance to other antibiotics. The least resistance was observed against norfloxacin, gentamicin, and streptomycin, indicating their higher *in vitro* effectiveness. Diverse resistance patterns were observed among the isolates (Figure 2B). A total of 44 multidrug-resistant isolates were selected for further study.



S.No.	Antibiotics	Total no. of resistant strain	Resistant percentage (%)
1	Ampicillin	70	100
2	Amoxicilin	70	100
3	Rifampicin	57	82
4	Erythromycin	51	73
5	Nalidixic acid	40	58
6	Co-trimoxazole	32	45
7	Lomefloxacin	20	29
8	Azithromycin	17	24
9	Tetracycline	16	23
10	Chloramphenicol	9	13
11	Nitrofurantoin	7	10
12	Kanamycin	5	7
13	Streptomycin	4	6
14	Gentamicin	1	1.5
15	Norfloxacin	1	1.5

Figure 2: Antibiotic susceptibility testing of bacterial isolates by the disc diffusion assay. Panel A shows the total number of antibiotics used in the study, while Panel B shows the percentage of resistant bacterial isolates observed against the tested antibiotics.

MIC determination in Gram-negative isolates

The minimum inhibitory concentration (MIC) of the test isolates was determined against seven antibiotics: chloramphenicol, ampicillin, tetracycline, norfloxacin, azithromycin, Cefotaxime, and nalidixic acid using the spot plate method. Among these, chloramphenicol was the most effective, inhibiting the isolates even at low concentrations (Supplementary Table 3).

Test isolates designation	**Minimum inhibitory concentration (µg/ml)**						
	Ampicillin	**Tetracycline**	**Norfloxacin**	**Azithromycin**	**Ciprofloxacin**	**Nalidixic acid**	**Chloramphenicol**
JSD-1	62.5	>500	>1250	6.25	31.25	250	>200
SAS5	62.5	>500	>1250	31.25	6.25	>250	>200
JSD-3	62.5	>500	>1250	>1250	>1250	>250	10
JSD-4	62.5	>500	>1250	6.25	625	1.25	>200
JSD-5	>250	50	>1250	>1250	>1250	>250	40
JSD-6	62.5	>500	>1250	31.25	6.25	1.25	20
JSD-12	>250	>500	>1250	312.5	312.5	>250	10
JSD-13	>250	250	>1250	312.5	6.25	>250	>200
JSD-14	>250	500	>1250	62.5	>1250	>250	2
JSD-16	>250	2.5	>1250	62.5	>1250	>250	2
JSD-17	>250	12.5	62.5	312.5	>1250	62.5	10
JSD-18	>250	250	62.5	312.5	6.25	62.5	>200
JSD-22	62.5	>500	>1250	6.25	6.25	>250	>200
JSD-26	>250	>500	>1250	>1250	>1250	>250	>200
JSD-27	>250	>500	>1250	1250	>1250	>250	200
JSD-28	>250	>500	>1250	>1250	>1250	250	10
JSD-30	>250	500	625	31.25	312.5	1.25	100
JSD-31	>250	12.5	625	6.25	6.25	62.5	>200
HCO-4	>250	50	1250	31.25	1250	250	2
HC0-5	>250	250	62.5	>1250	1250	62.5	20
HC0-6	>250	>500	>1250	6.25	31.25	>250	10
HCO-7	>250	>500	>1250	31.25	31.25	>250	40
USP-1	>250	>500	>1250	6.25	31.25	>250	100
USP-2	>250	25	>1250	312.5	625	>250	10
USP-6	>250	>500	>1250	312.5	625	>250	100
USP-8	>250	500	62.5	>1250	31.25	>250	10
USP-9	>250	250	31.25	625	>1250	>250	>200
USP-10	>250	50	31.25	31.25	>1250	>250	100
USP-11	>250	>500	31.25	31.25	>1250	1.25	10
USP-12	>250	>500	31.25	31.25	>1250	62.5	20
USP-13	>250	>500	625	31.25	625	62.5	40

Citation: Iqbal Ahmad*., et al.* "Elucidating the Antibacterial and Antibiofilm Efficacy of *Carum copticum* Against Multidrug-Resistant Bacteria of Wastewater Origin". *Acta Scientific Microbiology* 9.7 (2026): 29-43.

USP-14	>250	>500	1250	6.25	>1250	12.5	20
TCS-1	>250	50	1250	31.25	625	>250	20
TCS-3	62.5	>500	1250	6.25	6.25	>250	20
TCS-4	>250	>500	>1250	1250	6.25	>250	200
TCS-5	>250	50	>1250	1250	625	62.5	200
TCS-6	>250	125	31.25	1250	>1250	250	20
TCS-7	>250	50	31.25	>1250	>1250	>250	20
TCS-8	>250	500	1250	>1250	62.5	250	100
TCS-9	>250	50	>1250	312.5	6.25	62.5	20
TCS-11	>250	>500	312.5	312.5	62.5	>250	40
TCS-12	>250	>500	>1250	1250	62.5	>250	>200
TCS-14	>250	250	>1250	312.5	62.5	>250	>200
TCS-15	>250	12.5	>1250	1250	62.5	>250	>200

Supplementary Table 3: Minimum inhibitory concentration (MIC) of selected antibiotics against the test isolates.

β-Lactamase production in isolated Gram-negative bacteria

β-lactamase production was assessed using penicillin and ampicillin as substrates. Out of 44 test isolates, 31 hydrolyzed penicillin, 24 hydrolyzed ampicillin, and 24 isolates hydrolyzed both antibiotics, indicating widespread β-lactamase activity among the Gram-negative isolates (Table 1).

Bacteria	Test isolates designation	Substrate hydrolysis pattern	
		Penicillin	Ampicillin
E. coli	TCS-12	+	+
	JSD-12	+	+
	USP-9, TCS-1	+	+
	SAS5, JSD-30, JSD-31	+	+
Salmonella sp.	JSD-17, JSD-18, TCS-9, TCS-14	+	+
Pseudomonas sp.	JSD-13, HCO-4, USP-1	+	+
	TCS-7	-	+
Klebsiella sp.	TCS-11	+	+
	USP-14	+	-
Shigella sp.	USP-10	+	-
Serratia sp.	HCO-6, HCO-7	+	-
Proteus sp.	JSD-26	+	+
	JSD-5, JSD-6, JSD-28, TCS-3	-	-
Citrobacter sp.	USP-6, USP-12, TCS-5, TCS-4, USP-8	+	+
	HCO-5, USP-2, USP-13, TCS-6, TCS-8, TCS-15	+	-
	JSD-3, JSD-4, JSD-22	-	-
Enterobacter sp.	JSD-16, JSD-27, USP-11	+	+
	JSD-14	+	-
	JSD-1	-	-

Table 1: Distribution of β-lactamase activity among different MDR bacterial isolates based on penicillin and ampicillin hydrolysis patterns.

(+) Indicates hydrolysis of antibiotic used as substrate; (−) indicates no hydrolysis of antibiotic.

Detection of ESBLs in Gram-negative isolates

Isolates positive for both penicillin and ampicillin hydrolysis were further screened for extended-spectrum β-lactamase (ESBL) production. ESBL detection was performed using an initial phenotypic screening with cefotaxime (30 µg) disks, followed by a confirmatory phenotypic test with cefotaxime–clavulanic acid combination disks. Among the 24 potential β-lactamase producers, 10 isolates were confirmed as ESBL producers, with inhibition zone differences of ≥5 mm (Table 2).

Bacteria	**Test isolates designation**	**Zone of inhibition (mm)**		**Difference in zone diameter (mm)**	**Inference**	**Percentage of positive isolates within each group**
		Cefotaxime^{30}	**Cefotaxime^{30} + Clavulanic acid^{10}**			
E. coli	TCS-12	18	24	6	+	
	JSD-12	14	22	8	+	
	USP-9,	15	20	5	+	
	TCS-1	16	24	8	+	
	SAS5,	17	22	5	+	
	JSD-30	16	23	7	+	
	JSD-31	18	23	5	+	100.00
Salmonella sp.	JSD-17	14	20	6	+	50.00
	JSD-18	16	18	2	-	
	TCS-9	22	29	7	+	
	TCS-14	R	R	0	-	
Pseudomonas sp.	JSD-13	15	21	6	+	33.33
	HCO-4	15	16	1	-	
	USP-1	R	R	0	-	
Klebsiella sp.	TCS-11	R	R	0	-	0.00
Proteus sp.	JSD-26	R	10	4	-	0.00
Citrobacter sp.	USP-6	R	R	0	-	0.00
	USP-12	16	19	3	-	
	TCS-5	R	9	3	-	
	TCS-4	R	R	0	-	
	USP-8	R	R	0	-	
Enterobacter sp.	JSD-16	16	18	2	-	0.00
	JSD-27	17	19	2	-	
	USP-11	13	16	3	-	

Table 2: Phenotypic detection of extended-spectrum β-lactamase (ESBL) production among bacterial isolates using cefotaxime and cefotaxime–clavulanic acid combination discs. Mean zone of inhibition is from a set of triplicates expressed in mm.

[R indicates Resistant (no zone of inhibition), + indicates ESBL producer, − indicates non-producer].

Biofilm formation in Gram-negative isolates

Biofilm formation was assessed in 44 multidrug-resistant (MDR) Gram-negative isolates using the microtiter plate assay (Figure 3). The results revealed considerable variability in biofilm-forming ability, ranging from strong to weak producers. Among the tested isolates, *Escherichia coli*, *Enterobacter* sp., and *Serratia* sp. exhibited strong biofilm-forming capacity (Figure 3B, A, and D). *Pseudomonas* spp. demonstrated a moderate level of biofilm formation (Figure 3F). In contrast, *Salmonella* spp. displayed poor biofilm formation (Figure 3E), while *Proteus* spp. was categorized as weak biofilm producers (Figure 3G). The observed differences in biofilm formation may be attributed to variations in genetic traits, nutrient availability, and environmental conditions.

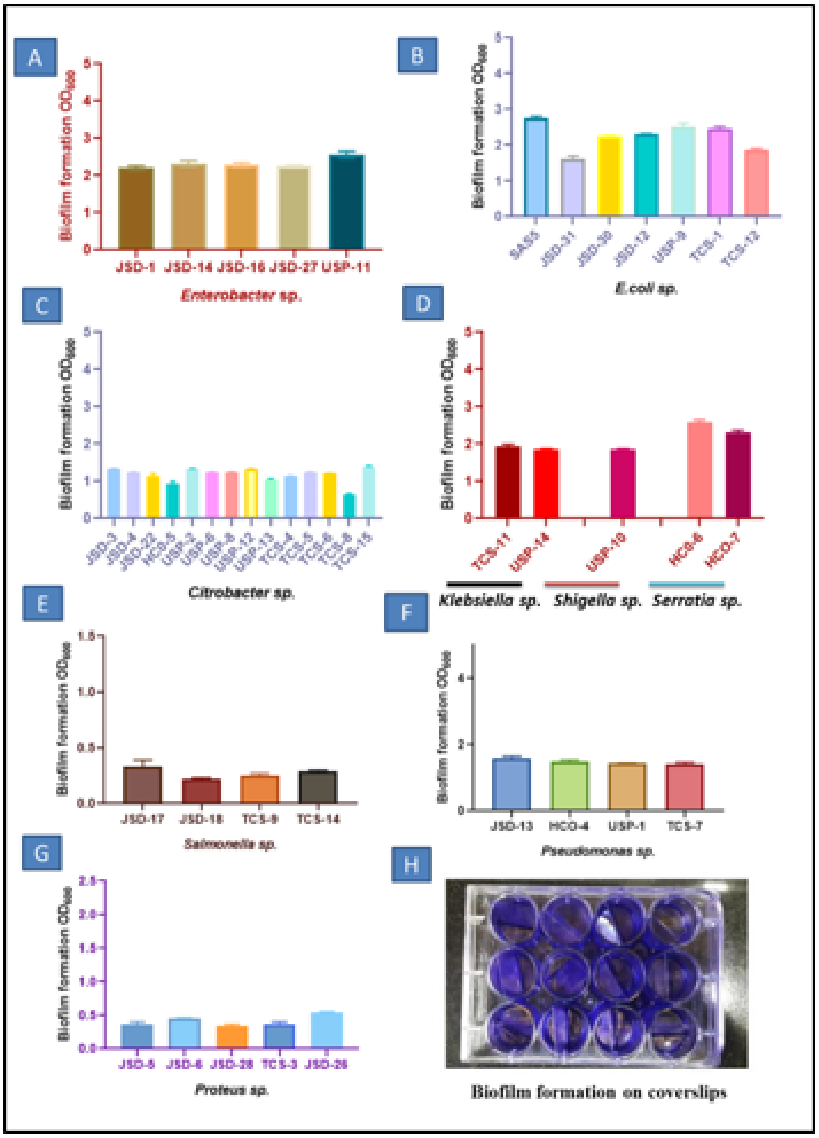


Figure 3: Biofilm formation by the isolated bacterial strains. Panel A shows *Enterobacter spp*. Panel B shows *Escherichia coli*; Panel C shows *Citrobacter spp*. Panel D shows *Klebsiella spp*., *Shigella spp*., and *Serratia spp*. Panel E shows *Salmonella spp*. Panel F shows *Pseudomonas spp*. Panel G shows *Proteus spp*., and Panel H shows biofilm formation on glass coverslips.

Antibacterial activity of plant extract

Preliminary screening of antibacterial activity was carried out using selected plant extracts at low concentrations against strong biofilm-forming *E. coli* strains (SAS5, JSD-30, and USP-9) and standard strains (*E. coli* ATCC 25922, *Staphylococcus aureus* MTCC 737, and *Pseudomonas aeruginosa* PAO1) by the disk diffusion method (Table 3). Based on the results, two extracts, *Carum copticum and Terminalia chebula*, showing higher activity were selected for further analysis. Their antibacterial efficacy was subsequently evaluated using the well-diffusion method at various concentrations against test isolates and standard strains (Table 4). It was observed that the *Carum copticum* extract exhibited greater activity against Gram-negative *E. coli* and Gram-positive *Staphylococcus aureus* than *Terminalia chebula.*

Mean zone of inhibition (ZOI) is from a set of triplicates expressed in mm							
S. No.	**Plant extracts**	***E. coli* test isolates**			***E. coli* ATCC 25922**	***S. aureus* MTCC 737**	***P. aeruginosa* PAO1**
		USP-9	**SAS5**	**JSD-30**			
1	*Cyperus scariosus*	-	-	-	-	13	-
2	*Syzygium cumini*	-	-	-	-	12	-
3	*Hemidesmus indicus*	-	-	-	-	13	-
4	*Holorrhena antidysentrica*	-	-	-	-	12	-
5	*Terminalia chebula*	-	12	-	13	13	6
6	*Carum copticum*	12	13	12	14	14	10
7	*Embeli ribes*	-	-	-	-	12	-
8	*Commiphora wightii*	-	-	-	-	12	-
9	*Carum copticum*	-	-	-	-	11	-

Table 3: Antibacterial activity of plant extracts at low concentrations determined by the disc diffusion method against standard and strong biofilm-forming test isolates.

Mean zone of inhibition (ZOI) is from a set of triplicates expressed in mm										
Test isolates	***Carum copticum* (µg/ml)**					***Terminalia chebula* (µg/ml)**				
	100	**200**	**400**	**800**	**1000**	**100**	**200**	**400**	**800**	**1000**
SAS5	7	10	13	17	20	7	7	11	15	18
USP-9	6	9	12	18	19	4	8	10	16	17
JSD-30	5	8	14	17	18	7	10	11	15	16
E. coli 25922	6	12	13	19	22	5	11	15	19	20
S. aureus MTCC 737	6	11	15	16	21	7	11	14	20	21
P. aeruginosa PAO1	5	9	11	17	19	6	8	10	16	20

Table 4: Antibacterial activity of different concentrations of *Carum copticum* and *Terminalia chebula* determined by the well diffusion method.

Antibiofilm activity of plant extract

The antibiofilm activity of the *Carum copticum* was evaluated at sub-MIC concentrations against selected test strains using the microtitre plate method (Figure 4). The extracts showed a dose-dependent reduction in biofilm formation, with maximum inhibition at the MIC/2 concentration of *Carum copticum*, corresponding to approximately 69.3% inhibition. Further, the reduction in biofilm formation was confirmed by SEM analysis (Figure 5).

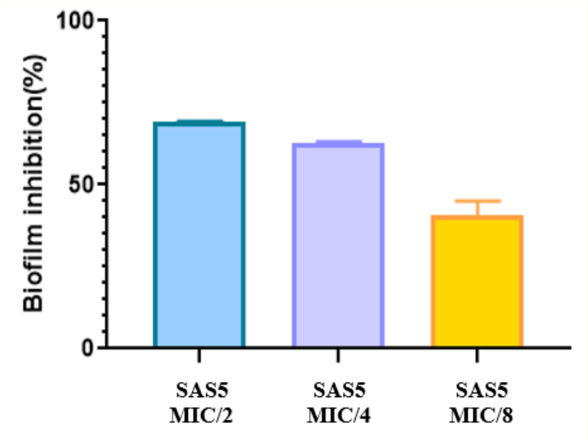
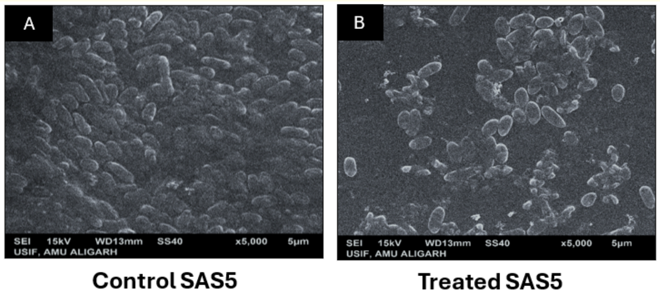


Figure 4: Percentage inhibition of biofilm formation in strain SAS5 by *Carum copticum* at different sub-MIC concentrations.



Control SAS5

Treated SAS5

Figure 5: Inhibition of the SAS5 strain biofilm by *Carum copticum* at MIC/2 concentration. Panel A shows the untreated control SAS5 biofilm, while Panel B shows biofilm inhibition by *Carum copticum* extract.

Discussion

This investigation revealed a high incidence of multidrug-resistant Gram-negative bacteria in wastewater samples, corroborating previous findings that wastewater settings operate as hotspots for the spread of antimicrobial resistance [20]. The elevated resistance to ampicillin and amoxicillin reported here aligns with other research indicating extensive β-lactam resistance among Enterobacteriaceae linked to wastewater [21]. Comparable resistance patterns were likewise documented in wastewater isolates from Aligarh city [13]. The detection of ESBL-producing isolates in this study further substantiates the increasing environmental spread of β-lactamase-mediated resistance mechanisms. ESBL-producing *E. coli* and associated Gram-negative bacteria have been increasingly identified in hospital and municipal wastewater systems globally [22]. About one-third of the isolates in this investigation demonstrated significant biofilm-forming capacity, potentially leading to increased antibiotic resistance and environmental durability. Comparable findings have been reported in environmental MDR bacterial isolates, in which biofilm formation significantly contributes to chronic survival and the development of resistance [23]. Cells associated with biofilms exhibit greater resistance to antibiotics than planktonic cells, due to the extracellular matrix impeding antimicrobial penetration. Among the assessed medicinal plant extracts, *Carum copticum* exhibited significant antibacterial and antibiofilm properties against the robust biofilm-forming *E. coli* isolate SAS5. Previous studies have shown that *Carum copticum* extracts exhibit significant antibacterial activity against Gram-negative organisms, attributed to thymol and carvacrol [11,24]. The dose-dependent decrease in biofilm formation observed in this study aligns with the findings by [25], which reported that phytoextracts and their compounds can disrupt biofilm and efflux pump mechanisms in MDR bacteria. The SEM examination further validated a significant alteration in biofilm structure following treatment with *Carum copticum* extract. Comparable structural degradation and reduced extracellular matrix production have been previously documented in bacterial biofilms treated with plant extracts [26]. The overall findings indicate that medicinal plants, particularly *Carum copticum*, can offer viable solutions for managing biofilm-associated infections and may be used as an alternative to antibiotic therapy.

Conclusions

The present study demonstrated that wastewater harbors multidrug-resistant, β-lactamase- and ESBL-producing Gram-negative bacteria with significant biofilm-forming ability, particularly *E. coli*, *Enterobacter*, and *Serratia* spp. Among the tested medicinal plants, *Carum copticum* exhibited the strongest antibacterial and antibiofilm activity against the MDR *E. coli* strain SAS5, with nearly 69.3% biofilm inhibition at the MIC/2 concentration, as confirmed by SEM analysis. The findings highlight the potential of *Carum copticum* as a promising natural agent against biofilm-associated MDR pathogens.

Author Contributions

- Rahisuddin Khan: Experimental work and manuscript writing
- Shirjeel Ahmad Siddiqui: Manuscript writing.
- Vishnu A. L.: Manuscript writing,
- Iqbal Ahmad: Study plan and guidance.

Data Availability Statement

The data supporting the findings of this study are available within the manuscript and its supplementary materials.

Acknowledgments

The authors are thankful to the University Sophisticated Instrumental Facility (USIF), AMU, Aligarh, for providing the required facilities.

Funding

The author(s) reported there is no funding associated with this work.

Conflict of Interest

The authors declare no conflict of interest.

Bibliography

1. World Health Organization. "Antimicrobial Resistance: Global Report on Surveillance". WHO Press (2014).
2. Chaudhary Nikita., *et al*. "Heavy Metals and Emerging Organic Pollutants in Hospital Wastewater and Domestic Sewage in Aligarh City: An in vitro Analysis of Toxicity and Modulatory Effects on Biofilm Development". *Water, Air, and Soil Pollution* 237.8 (2026): 502.
3. Larsson DG Joakim and Carl-Fredrik Flach. "Antibiotic Resistance in the Environment". *Nature Reviews Microbiology* 20.5 (2022): 257-269.
4. Karkman Antti., *et al*. "Antibiotic-Resistance Genes in Waste Water". *Trends in Microbiology* 26.3 (2018): 220-228.
5. Bush Karen and Patricia A Bradford. "β-Lactams and β-Lactamase Inhibitors: An Overview". *Cold Spring Harbor Perspectives in Medicine* 6.8 (2016): a025247.
6. Rawat Deepak and Devaki Nair. "Extended-Spectrum β-Lactamases in Gram Negative Bacteria". *Journal of Global Infectious Diseases* 2.3 (2010): 263-274.
7. Hall Stoodley P and T F Mah. "Molecular Mechanisms of Biofilm-Based Antibiotic Resistance". *Future Microbiology* 12.10 (2017): 881-903.
8. Ahmad Iqbal., *et al*. "Environmental biofilms as reservoir of antibiotic resistance and hotspot for genetic exchange in bacteria". Beta-Lactam Resistance in Gram-Negative Bacteria: Threats and Challenges. Singapore: Springer Nature Singapore (2022): 237-265.
9. Ahmad Iqbal., *et al*. "Cultural and molecular approaches to analyse antimicrobial resistant bacteria from environmental samples". *Microbial Diversity in the Genomic Era. Academic Press* (2024): 759-776.
10. Siddiqui Shirjeel Ahmad and Iqbal Ahmad. "Harnessing anti-infective efficacy of Cinnamomum verum in synergy with β-lactam and fluoroquinolones drugs to combat virulence and biofilms of Pseudomonas aeruginosa PAO1". *Microbial Pathogenesis* 197 (2024): 107097.
11. Maheshwari Meenu., *et al*. "Bioactive extracts of Carum copticum and thymol inhibit biofilm development by multidrug-resistant extended spectrum β-lactamase producing enteric bacteria". *Biofouling* 35.9 (2019): 1026-1039.
12. Ananthanarayanan R and CK Jayaram Paniker. "Textbook of Microbiology". 6th ed., Orient Longman Private Ltd. (2000): 7-22.
13. Chaudhary N., *et al*. "Prevalence of Biofilm-Forming, ESβLs and Metallo-β-Lactamase Producing Gram-Negative MDR Bacteria in the Domestic and Hospital Wastewater of Aligarh City". *Current Microbiology* 82.8 (2025): 354.
14. Bauer A W., *et al*. "Antibiotic Susceptibility Testing by a Standardized Single Disk Method". *American Journal of Clinical Pathology* 45.4 (1966): 493-496.
15. Clinical and Laboratory Standards Institute. "Performance standards for antimicrobial susceptibility testing". 27th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute (2017).

16. Perret CJ. “Iodometric Assay of Penicillinase”. *Nature* 174.4439 (1954): 1012-1013.
17. Catlin BW. “Iodometric Detection of Haemophilus influenzae Beta-Lactamase: Rapid Presumptive Test for Ampicillin Resistance”. *Antimicrobial Agents and Chemotherapy* 7.3 (1975): 265-270.
18. Qais F A., *et al*. “Interference of Quorum Sensing Regulated Bacterial Virulence Factors and Biofilms by Plumbago zeylanica Extract”. *Microscopy Research and Technique* 84.12 (2021): 3150-3160.
19. Perez C., *et al*. “An Antibiotic Assay by the Agar-Well Diffusion Method”. *Acta Biologiae et Medicinae Experimentalis* 15 (1990): 113-115.
20. Manaia Célia M., *et al*. “Antibiotic Resistance in Urban Aquatic Environments: Can It Be Controlled?” *Applied Microbiology and Biotechnology* 103.4 (2019): 1543-1557.
21. Ahmed Vikar., *et al*. “Pathogenic potential of amoxicillin-clavulanic acid resistant Klebsiella pneumoniae isolated from aquatic environment: a study of multidrug resistance and virulence”. *BMC Infectious Diseases* 25.1 (2025): 1495.
22. Buelow E., *et al*. “The Abundance of Antibiotic Resistance Genes in Hospital Wastewater and the Surrounding Environment”. *PLoS ONE* 13.4 (2018): e0194690.
23. Jamal Muhsin., *et al*. “Bacterial biofilm and associated infections”. *Journal of the Chinese Medical Association* 81.1 (2018): 7-11.
24. Bhardwaj Mona., *et al*. “Phytochemical Compounds and Antimicrobial Activity of Carum copticum”. *Journal of Applied Pharmaceutical Science* 6.3 (2016): 157-162.
25. Samreen., *et al*. “Efflux pump inhibition-based screening and anti-infective evaluation of Punica granatum against bacterial pathogens”. *Current Microbiology* 81.1 (2024): 51.
26. Singh Rajesh., *et al*. “Antibiotic Resistance Pattern among Environmental Bacterial Isolates from Wastewater”. *Environmental Monitoring and Assessment* 192 (2020): 120.