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Combined Effect of Isolated Bacteriophage and Neem Extract on Isolated Multiple Drug-Resistant Pathogenic *Escherichia coli* E1 From Well Water

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ABSTRACT: Multiple drug-resistant *Escherichia coli* (*E. coli*) is a serious cause of concern, and they can be observed in hospital settings, natural environment, and animals. Dissemination of multiple drug-resistant (MDR) *E. coli* can pose a high risk to public health. Moreover, they are hard to control with commercial antibiotics, since they have acquired resistance against most of them. Therefore, to control multiple drug-resistant bacteria, alternative strategies have been adopted such as phage therapy, herbal remedies, nanoparticles etc. In the current study, the combined application of neem leaf extract and bacteriophage is used to control an isolated multiple drug-resistant *E. coli* E1. We have applied 0.1 mg/ml concentration of neem extract in combination with an isolated phage vB_EcoM_C2 of 10^{11} titer and found that the combinatorial treatment approach significantly controls the growth of *E. coli* E1 as compared to a single non-combinatorial treatment. In this study, every *E. coli* cell is targeted by 2 antimicrobials (phage and neem extract) at the same time, which is more effective as compared to the sole treatment. Implementation of the neem extract with phage opens a new alternative approach to the chemotherapeutics for the control of multiple drug-resistant bacterial pathogens. This approach may be effective, economical, and eco-friendly to combat MDR.

KEYWORDS: Bacteriophage, neem extract, quorum sensing, *E. coli*, multiple drug resistance

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Introduction

E. coli is a leading cause of community-acquired as well as nosocomial infections in developing countries. It causes dysentery, urinary tract infections, and septicemia in humans and animals. *E. coli* is commonly transmitted through contaminated food and water.[1] The emergence of drug resistance in *E. coli* has created a high risk to immunocompromised hosts.[2-4] They have acquired various mechanisms to thrive in adverse environmental milieu.[5] The infection caused by multiple drug-resistant (MDR) *E. coli* can be a cause of mortality and morbidity, and is responsible for expansive hospital expenditure as well.[6] They can share resistance genes very frequently with other bacterial species, and these genes can be acquired by human and animal microflora.[7] Colonization of MDR in the gastrointestinal tract can lead to severe ailments in the body. It has been documented that they have evolved resistance mechanisms against the extended spectrum of β-lactam antibiotics; hence it is a serious matter of concern.[8] There is no new drug currently available to control such MDR *E. coli*, therefore, to control the MDR *E. coli* various remedies have been adopted, such as lactoferrins, nanoparticles, bacteriophages, plant-based antimicrobials etc.[9,10] Bacteriophage therapy is not very new; it has been in medical practices since the early phase of the 19th century, later, the discovery of antibiotics faded the spark of phage therapy. Phages are obligate parasites of bacteria and are specific to their hosts, therefore, they have been reintroduced in medical practices to control the MDR pathogens. The specific properties of phages such as self-replicating efficiency, eco-friendly nature, pharmacokinetics, and pharmacogenic make them ideal alternatives to antibiotics. Nevertheless, bacteria have evolved resistance mechanisms against bacteriophages,[11,12] and as a result now phage therapy has limited efficacy. Therefore, phage therapy along with other antimicrobials has been studied by several research groups to control the superbugs.[13] In our earlier study, we reported the control of *P. aeruginosa* by combined application of ciprofloxacin antibiotic and isolated phages.[14] In the current study, we have further extended the approach and applied isolated bacteriophages vB_EcoM_C2 along with neem extract against MDR *E. coli* E1. Neem extract exhibits scavenging properties for the free radicals and can-do repair DNA damage, alter cell cycle, programed cell death, mitigation autophagy, and anti-inflammatory.[15-17] Neem-based products are safe and have abroad impact on health both for humans and livestock. After extensive research on the different parts of the neem plant, it has been identified that the active component of the neem tree is phenolic components along with nimbin (triterpene), which has been carrying a broad range of activities such as antimicrobial, antipyretic, antiseptic, antihistamines and fungicidal. It is also associated with anti-inflammatory and antioxidant properties that reduce cell damage by reactive oxygen species.[18]

Therefore, in our study, we have applied 0.1 mg/ml concentration of neem extract in combination with an isolated phage vB_EcoM_C2 of 10^{11} titers and found that the combinatorial treatment approach significantly controls the growth of *E. coli*

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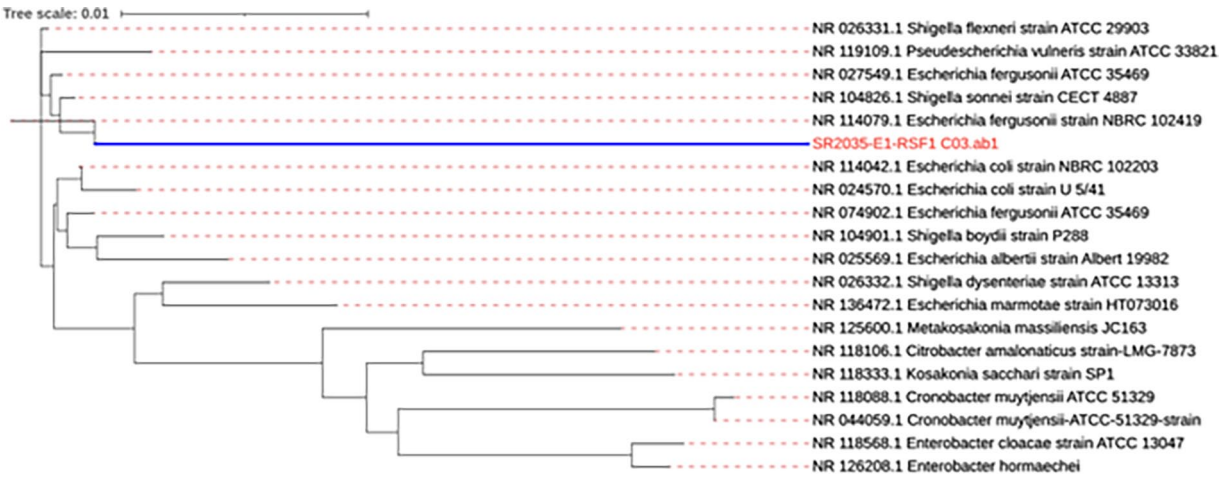


Figure 1. Identification of isolated bacteria.
Phylogenic tree of 16s rRNA sequence of isolated bacteria that is showing 98% similarity with *E. coli*. The sequence has been submitted in GenBank and accession no. is MW590616.

E1 as compared to the sole treatment. Implementation of neem extract with phage opens a new alternative chemotherapeutics approach to control multiple drug-resistant bacterial pathogens.

Materials and Methods

Microbes and chemicals

E. coli was isolated from well water, Kozhikode, Kerala, India. The culture was grown on McConkey media at 37°C and maintained in 50% of glycerol stock at −20°C for further use. Bacteriophages were isolated from the Canoli canal and hospital around the Kozhikode city, Kerala, India, and maintained in SM buffer (HiMedia, India) at −20°C for further study. All the chemicals and buffers were purchased from HiMedia and Merck.

Isolation and identification of E. coli E1

E. coli E1 was isolated from the well water by using the serial dilution method. Briefly, 1 ml of sample was taken and serially diluted, and the spreading method was performed on chromocult media and EMB by 1 ml of sample. Plates were incubated at 37°C for 24 hours. After incubation, colonies were characterized and identified by using biochemical tests and the 16S rRNA molecular sequencing method. After the 16S rRNA sequencing method, BLAST alignment was performed with existing sequences of bacteria using the NCBI (The National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov) database. The output Multiple Sequence Alignment Clustal Omega files used for phylogenetic tree preparation in iTOL: Interactive Tree Of Life (embl.de) online server. BLAST result shows 98% sequence similarity with *E. coli* bacteria therefore confirms the isolated species is *E. coli*. 16S rRNA Sequence has been submitted to GenBank with accession no. MW590616 (Figure 1).

Antibiotic susceptibility test

The antibiotic susceptibility test was performed as per the Kirby Bauer disk diffusion method according to the clinical and laboratory standards institute (CLSI), 2015 guideline.[19] Briefly, the overnight culture of *E. coli* E1 with 0.1 OD was taken and spread on the MHA media (Mueller Hinton Agar) poured plate, further the antibiotics disks (CXT-30mcg), erythromycin (ERT-15mcg), teicoplanin (TEI-30mcg), clindamycin (CLD-2mcg), linezolid (LZD-30mcg), aziythromycin (AZM-15mcg), cefpirome (CFP-30mcg), ceftazidime (CFM-30mcg), imipenem (IPM), piperacillin (PIT-10U), colistin (CLS-10mcg), and vancomycin (VAN-30mcg) were placed on the plate and incubated at 37°C for 18 hours. After incubation, the diameter around the disk was measured.

Blood Agar test

The blood agar test was performed as per the Buxton protocol,[20] with some modifications. Five percent sheep blood was added to Trypton Soy Agar media after autoclaving. Prepared media was poured into a sterilized petri dish and then *E. coli* E1 was streaked on the media. The culture plates were incubated at 37°C for 24 hours. After incubation, the clear zone was observed for beta hemolytic activity.

Congo red binding assay

Congo red binding assay was performed as per the protocol of Chen et al[21] with some modifications. 0.3% congo red dye was added to BHI (brain heart infusion) media supplemented with 1% starch. After autoclaving of media, plates were prepared by using congo red added BHI agar media, and streaking was performed with culture was incubated for 24 hours at 37°C. After incubation, dark red to the blackish red colony was observed for determination of the presence of amyloid fibers in bacteria.

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HPLC for the detection of quorum sensing molecules of E. coli E1

HPLC was done to determine the quorum-sensing signaling molecules in the bacteria. To perform the analysis, a C18 (50 × 4.6 mm) column was used. Shimadzu class VP V 6.13 SPI HPLC was used for purification. The box temperature was maintained at 25°C to carry out the analysis. The run was performed in 70% acetonitrile mobile phase, which was prepared in MQ water. C6-HSL at 1.0 ng/ml (dissolved in methanol) was used as a standard in this analysis. The column flow rate was at 2 ml/min, and the wavelength was set at 210 nm. The 20 µl sample was injected. Data analysis was performed by using shimadzu hplc class vp software.

Bacteriophage isolation

For phage isolation, sewage water and canoly canal water were chosen. Isolation was performed as per Yazdi et al[22] with some modifications. 10 ml of water sample was taken and centrifuged at 12 000×*g* for 10 minutes, then the supernatant was removed and filtered through a sterilized 0.45 µm pore size syringe filter. The filtrate was mixed with 10 ml of log phage *E. coli* E1 and incubated it for 24 hours at 37°C. Furthermore, to check the presence of phages in lysate, the earlier process was repeated, the lysate was centrifuged and filtered then a double layer agar assay was performed. To perform the double layer agar assay, hard agar plates were prepared, and the top agar layer was overlaid with a mixture of 6 ml of 0.6% soft agar, 100 µl of filter sterilized water sample, and 100 µl of host sample. After pouring the top layer, plates were incubated overnight at 37°C. The clear zone of plaque was observed the next day.

Spot assay

The spot assay was done to determine the lytic activity of isolated bacteriophages vB_EcoM_C2. For the spot test, hard agar plates were prepared followed by overlaid with 5 ml of 0.6% of agar mix with 100 µl of *E. coli* E1. After pouring of soft agar layer, the spotting was done by using 10 µl of isolated phage vB_EcoM_C2 and incubated for 24 hours at 37°C. After incubation, the clear zone was examined.

Electron microscopic analysis of isolated bacteriophagevB_EcoM_C2

Electron Microscopy of isolated phage vB_EcoM_C2 was done by using the negative staining method as per Goodridge et al[23] with some modifications. Purified 10 µl of phage vB_EcoM_C2 was placed on the copper-coated grid and dried for 5 minutes and then phosphotungstic acid (PTA) staining was applied for 20 seconds followed by 10 minutes air dry. The grid was observed under Transmission Electron Microscope (Thermo Fisher Scientific, Talos L120C) at 200 kv.

Isolation of bacteriophage vB_EcoM_C2 genomic DNA

Bacteriophage vB_EcoM_C2 genomic DNA was isolated as per Sambrook et al[24] with some modifications. Briefly, 5 ml of 10^{12} pfu of phage vB_EcoM_C2 lysate was taken and treated with 10 mM Tris-HCl, pH 7.0, 1.0 mM EDTA, 10 µl of proteinase K [20 mg/ml] and incubated in a water bath for 1 hour at 65°C. After incubation, the lysate was treated with an equal volume of tris saturated phenol, which was followed by the chloroform treatment. After chloroform treatment, the solution was centrifuged at 10 000×*g* for 20 minutes, top clear phase was recovered and treated with 100% ethanol and 3M sodium acetate, which was followed by 70% ethanol treatment. Extracted DNA was dissolved in TE buffer and run on the 1% agarose gel to identify the size of genomic DNA.

Determination of adsorption rate of isolated bacteriophage vB_EcoM_C2

Adsorption rate of isolated bacteriophage vB_EcoM_C2 was determined by using 1.0 multiplicity of infection (MOI) of phage vB_EcoM_C2 and host E. coli *E1*. Briefly, the frequency of adsorption of phage to host was observed every 15 minutes for 120 minutes by doing the double layer agar (DLA) method. In every 15 minutes, the un-adsorbed phages were counted by doing DLA for up to120min. DLA plates were incubated at 37°C for 24 hours. After incubation, the pfu/ml was determined.

Crude neem extracts preparation

The neem leaf samples were collected from the Center for Water Resources Development and Management (CWRDM) campus, Kozhikode, Kerala, India in June 2020. The leaves were separated from the stems and were dried at 40 to 45°C for 5 days. After drying, the leaves were ground by using a mixer grinder. Further, the leaf powder was used for extraction purposes. 100 gm of leaf powder was mixed with 100 ml of DMSO in a 500 ml soxhlet extraction chamber and the unit was assembled for the distillation process. Extraction was carried out for 8 hours and DMSO extraction was followed by the methanol extraction. The methanol extract was evaporated by using a rotary evaporator, and eventually, a semisolid crude was obtained after vacuum filtration. The crude neem extract was dried at 45°C and used for further analysis.

FTIR analysis of neem crude extract

FTIR analysis was performed to detect the presence of organic and polymeric functional groups in the neem extract. 1 ml of neem extract was taken for analysis and the change in the absorption pattern was analyzed. The range was kept between 4000 and 400 cm^{-1} by using Perkin Elmer FTIR spectrophotometer.

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Table 1. Determination of antibiotic susceptibility pattern of *E. coli* E1.

NAME OF TESTS / NAME OF ISOLATE	ANTIBIOTIC SUSCEPTIBILITY TEST	CONGO RED BINDING ASSAY	HEMOLYTIC TEST
E. coli	CX^R, IMP^R, CFT^R, CFM^R, PIC^R, CPM^R, CST^R, VAN^R, ERT^S, TEI^R, CLD^R, LZL^R, AZM^S, CFP^S	+++	+++ (β hemolytic test)

Abbreviations: R, resistance; S, sensitive; +, strongly positive.

Well diffusion assay for detection of antibacterial efficacy of neem extract

Well diffusion assay was performed as per the protocol of Dahiya and Purkayastha[25] with some modifications. In brief, overnight culture of *E. coli* E1 with OD 0.1 was spread on the nutrient agar plates. The wells were made with a sterile cork borer (6 mm) and further the different concentrations of neem extract were added into the wells. Plates were incubated at 37°C for 24 hours, after incubation the inhibition zone size was measured.

Combined application of phage vB_EcoM_C2 and neem extract on E. coli E1 biofilm formation

Phage vB_EcoM_C2 of 1×10^{11} pfu/ml was taken and added to neem extract in equal amount. Overnight culture of 1×10^6 of *E. coli* E1 was prepared. 100 µl log culture was added in a microtiter well in triplicates, which was followed by adding 100 µl mixture of phage vB_EcoM_C2 and neem extract into each well. After adding the phage vB_EcoM_C2 and neem extract, the treated cells were incubated for 24 hours at 37°C for the formation of biofilm. After incubation, the viability of biofilm cells was analyzed.

Viability test by using MTT dye

The MTT assay was performed to determine the viability of biofilm cells by using tetrazolium dye. A 0.3% solution of MTT dye was prepared in PBS buffer. A 200 µl of MTT dye solution was added in each well containing *E. coli* E1 cells treated with bacteriophages vB_EcoM_C2 and neem extract. After adding dye, incubation was done for 20 minutes in dark at room temperature. After incubation, the MTT dye was removed, and wells were washed with PBS buffer. 200 µl of DMSO (dimethyl sulfoxide) was added in the well and absorption was measured at 540 nm wavelength.

Total viable count of biofilm cells after combined treatment of phage vB_EcoM_C2 and neem extract

To determine the viability of *E. coli* E1 biofilm cells after the sole treatment of neem extract, phage vB_EcoM_C2 and combined treatment of vB_EcoM_C2 and neem extract, 100 µl of 1 mM Na-EDTA dissolved biofilm samples were taken from every treatment and spread on the trypton soy agar plate and incubated at 37°C for 24 hours. After incubation, colonies on the plates were counted and CFU/ml was determined.

Statistical analysis

All the experiments were done in triplicate and repeated 3 times to check the consistency of the test and the results are expressed as mean ± SD.

Results and Discussion

Isolation and Identification of bacteria

E. coli E1 was isolated from the well water, and it was identified through morphological, biochemical, and molecular methods. Colony morphology on EMB and chromocult selective media, partially characterized the organism belonging to *E. coli*. Biochemical tests were also conducted, and results indicated that the isolate belongs to *Enterobacteriaceae* (result not shown). BLAST alignment of 16S rRNA data also indicates that the isolated bacteriam is *E. coli* (Figure 1). The occurrence of *E. coli* in wastewater is very common in developing countries and has been reported in many studies.[2-4] Nevertheless, MDR *E. coli* have been frequently isolated from poultry, meat products, and vegetables.[26-28] The prevalence of drug resistance *E. coli* is a serious concern to public health. Odonkor, & Addo,[1] isolated *E. coli* from drinking water sources. However, in the present study, *E. coli* E1 was isolated from the drinking well water. The well water is frequently used for drinking, cooking, and cleaning purposes; hence, the occurrence of *E. coli* is an alarming sign for public health.

Determination of antibiotic susceptibility pattern of E. coli E1

The antibiotic-resistant pattern of *E. coli* E1 was identified by using antibiotics (mentioned in Table 1). Antibiotic susceptibility test confirmed that *E. coli* E1 was resistant to cefoxitin, imipenem, cefotaxime, ceftazidime, piperacillin, cefepime, colistin, vancomycin, teicoplanin, clindamycin, linezolid while sensitive to erythromycin, azithromycin, and cefpirome. The emergence of multiple drug-resistance in bacteria is very common. Resistance mechanisms in bacteria can be phenotypic, genetic, and spontaneous. The emergence of antibiotic resistance in bacteria is an evolutionary process that allows bacteria to survive under stress (antibiotic) conditions. Bacteria are

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Table 2. Identification of quorum sensing molecules in *E. coli* E1 by HPLC.

COMPOUND	RETENTION TIME
C6-HCL (Standard)	4.650 ± 0.025
E. coli E1 (Sample)	4.850 ± 0.117

± standard deviation.

using efflux pumps, enzymes, plasmids, and mobile genetic elements, to neutralize antibiotic stress; additionally, they are involved in the exchange of antibiotic-resistance genes among other species to make them fit in the environment.[29] The occurrence of ciprofloxacin and imipenem resistance in *E. coli* is a concerning issue, since 1987 no new antibiotic has been discovered and imipenem is the last line of the drug to the gram-negative bacteria.[30] In the current study, the isolated *E. coli* E1 was resistant to various classes of antibiotics including imipenem. If the imipenem MDR bacteria continue to spread, it could be challenging to control the infection using a commercially available antibiotic.

Congo red assay

Congo red binding assay showed the presence of amyloid fibers. These fibers are necessary for the curli formation and help in the adherence of bacteria to the surface which is followed by biofilm formation. The presence of a dark red color colony on the congo red agar plate indicates the presence of amyloid fibers (Table 1). Congo red binding assay clearly indicated that the isolated *E. coli* E1 is capable of producing amyloid fibers on the cell surface, which can be beneficial for the isolate to adhere and form biofilm.

Identification of quorum sensing molecules in isolated E. coli E1

Bacteria use quorum-sensing molecules for communication purposes. They use a quorum sensing molecule for biofilm formation, and it is a density-based mechanism. There are different types of quorum sensing signaling molecules that have been identified in various classes of bacteria. In *E. coli*, the LuxS/AI-2 quorum sensing system has been observed. This is governed by the C6 HSL molecules.[31] Therefore, in this study, HPLC was performed using C6 HSL as a standard (Table 2) (Supplemental Figures 5 and 6) for the identification of C6-HSL in isolate *E. coli* E1. In the HPLC chromatogram, the peak of C6-HSL was observed (Table 2), and the quantity of C6-HSL in the supernatant was seen approximately at 0.25 ng/ml. Hence, HPLC analysis confirmed that isolated *E. coli* E1 may be assisted by the C6-HSL type of signaling mechanism (quorum sensing). C6-HSL is required for biofilm formation and is also involved in the regulation of virulence factors in bacteria. Control of antibiotic resistant bacteria may be possible to some extent, but biofilm-forming MDR bacteria are very hard to control. Biofilm is a community of microbes encapsulated by exopolymeric substances (EPS). The microbes that are embedded in the biofilm are 1000 times more resistant to antibiotics than the free-living bacteria.[32] In the present study, the isolated *E. coli* E1 is a biofilm forming bacteria that has also shown drug-resistant characteristics. Hence, the control of biofilm-forming, antibiotic-resistant *E. coli* E1 is very challenging with commercially available antibiotics. Therefore, to control the drug-resistant biofilm forming in *E. coli* E1, we have adopted phage therapy.

Isolation and characterization of bacteriophages vB_EcoM_C2

We isolated bacteriophages vB_EcoM_C2 from the canoly canal water. Because we observed a clear zone around the spotted area against the host bacteria *E. coli* E1, the spot assay indicated that the isolated bacteriophage was assisted with lytic activity. Morphological characterization of the isolated bacteriophage was done by using transmission electron microscopy (TEM). The electron micrograph revealed that the isolated bacteriophage is a tailed bacteriophage with a hexagonal head (Figure 2a). The vB_EcoM_C2 is a double-stranded DNA phage with a genome size of about 6 kb, according to genomic DNA analysis of isolated bacteriophages (Supplemental Figure 7). For the identification of taxonomy of bacteriophage vB_EcoM_C2, we did a comparative study with previously reported *E. coli* specific bacteriophage, and electron micrograph of the current study; based on the comparative analysis, we assumed that the isolated bacteriophage may belong to *Myoviridea* .[33-42]

Further, the growth kinetics of isolated bacteriophage vB_EcoM_C2 were determined by the adsorption assay, which indicates the adsorption efficiency of isolated bacteriophage. We observed 10^8 pfu/ml of phage vB_EcoM_C2 within 15 minutes of the adsorption assay, and after 120 minutes we got 10^2 pfu/ml (Figure 2b). The adsorption assay indicated that the bacteriophage vB_EcoM_C2 has moderate adsorption efficiency. Generally, fast adsorption is associated with rapid infection of the host cell.

Antibacterial efficacy of neem leaves extract

A methanol extraction method was used to extract the organic components from neem leaves. The phenolic compounds, along with nimbin, are the most prominent components of neem extract, and they have high antimicrobial properties. According to Alzohairy,[43,44] the nimbin of neem exhibits a broad range of killing efficiency against a different class of bacteria. As a result, the extract was tested for antibacterial efficacy against the *E. coli* E1. In Figure 6 (provided in Supplemental data), a clear zone around the well was observed, the clear zone confirmed that neem extract has antimicrobial properties

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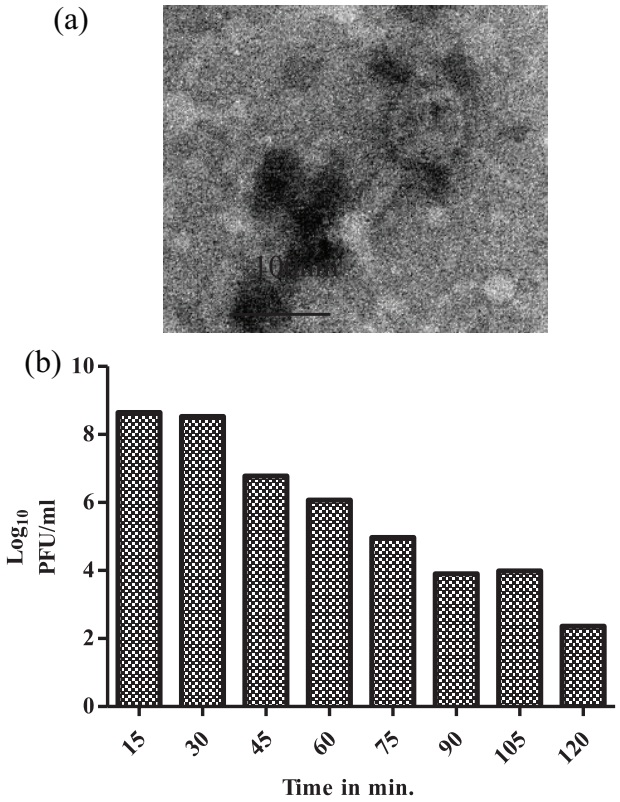


Figure 2. Determination of morphology and adsorption rate of isolated bacteriophage vB_EcoM_C2: 100 nm: (a) TEM image of *E. coli* E1 specific isolated bacteriophage vB_EcoM_C2 and (b) adsorption of *E. coli* E 1 phage at different time intervals in pfu/ml.

against *E. coli* E1. Further, the active component of neem extract was identified through Fourier transform infrared (FTIR) spectroscopy.

Identification of active component of neem extract by FTIR

FTIR analysis of neem leaf extracts reveals broad peaks at 3670 and 3350 cm^{-1}, corresponding to OH- stretching and indicating the presence of aromatic and primary alcohol in the extract.[38] A peak at 2910 cm^{-1} corresponds to alkenes, while a peak at 1320 cm^{-1} corresponds to an aromatic compound, and a peak at 1260 cm^{-1} represents a carboxylic compound.[45,46] We have identified similar types of peaks in our neem extract sample as well (Figure 3). According to Einhellig[47] and Pillingeret,[48] the presence of alcoholic and carboxylic groups in neem leaf extract is responsible for antimicrobial activity. In the current study, we have also observed the presence of carboxylic and alcoholic components in the neem extract, which might be responsible for the antimicrobial properties of the extract against *E. coli* E1.

Combined efficacy of neem extract and isolated bacteriophages vB_EcoM_C2 against E. coli E1 biofilm

The MTT assay was done to determine the viability of phage vB_EcoM_C2 and neem extract-treated biofilm cells. MTT

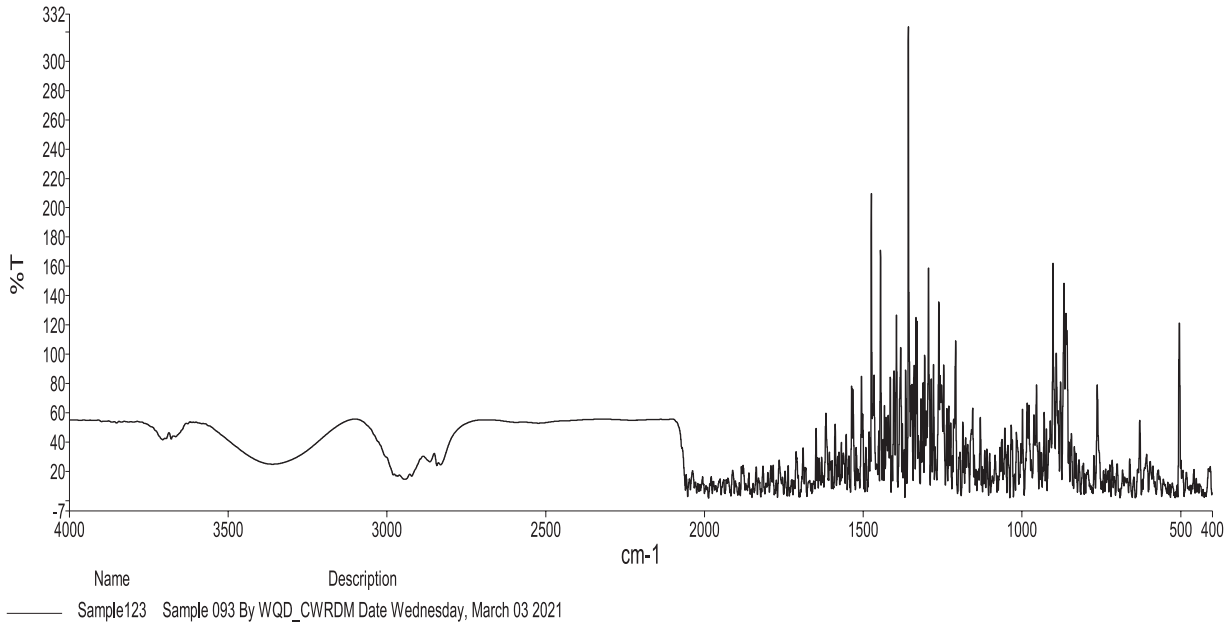


Figure 3. Identification of active component of neem extract by FTIR.
FTIR spectra show the presence of functional groups in neem extract, the highest peak representing the presence of alcoholic and carboxylic groups in the neem extract.

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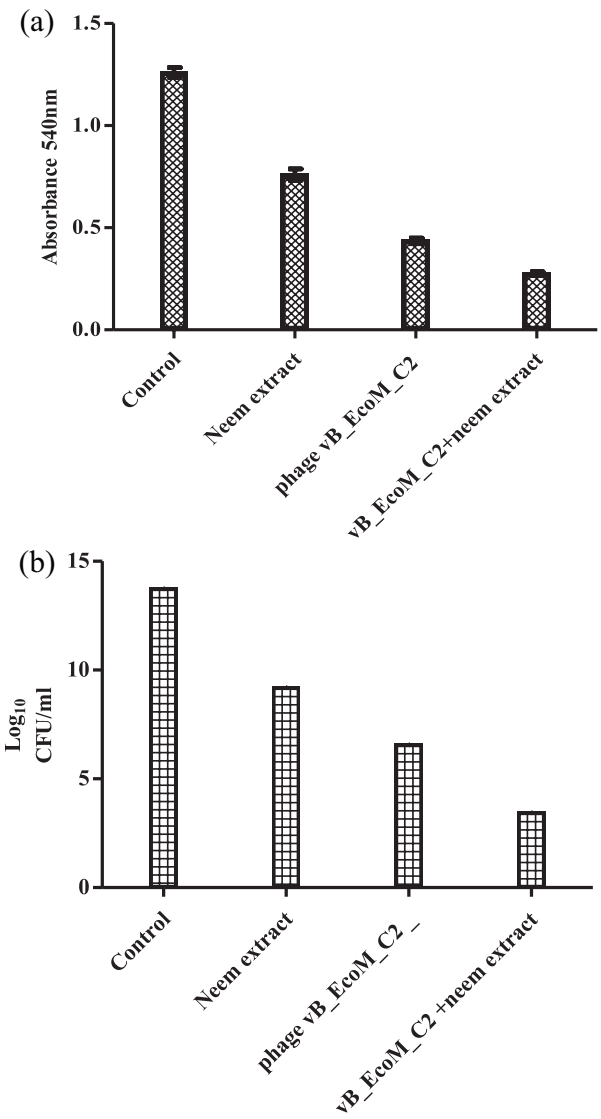


Figure 4. Combined efficacy of neem extract and isolated bacteriophages vB_EcoM_C2 (by MTT assay): (a) MTT assay of combined as well as sole treatment of isolated bacteriophage vB_EcoM_C2 and neem extract. (b) Determination of total viable count of control, neem extract treatment, phage vB_EcoM_C2 treatment and combined treatment of vB_EcoM_C2 and neem extract.

dye is a redox dye, and it is yellow in color. However, metabolically active cells (live) tend to turn the color from yellow to purple. We observed, up to 60% and 45% killing of *E. coli* E1 biofilm cells in the sole treatment of neem extract and phage vB_EcoM_C2 respectively (Figure 4a). Viazis,[13] has reported approximately 50% killing of *E. coli* EHEC O157:H7 strains in their study. Additionally, when Viazis,[13] applied the combination of phage cocktail and trans-cinnamon essential oil to the EHEC O157:H7 strain, they found that the killing efficacy was significantly increased. Wang et al,[49] applied isolated bacteriophages to control the *E. coli* O157:H7 strain in domestic ruminants, meat products, fruits, and vegetables. Rad et al,[50] observed approximately a 2-log CFU reduction in *E. coli* on the application of a phages cocktail. Malik et al[51] observed approximately 60% killing of E. coli clinical isolates, but the phage cocktail provided 86% killing activity.

Control of drug-resistant bacteria by phages has been in practice since the initial phase of the 19th century.[52] Nonetheless, one limitation of phage therapy is that bacteria may develop resistance mechanisms to them.[12] As a result, phages may be rendered ineffective in eliminating phage-resistant cells. In the current study, we found that phage vB EcoM C2 had a very low killing efficiency against E. coli E1, so we added neem extract to target the phage-resistant E. coli E1. The combination of neem extract and the vB_EcoM_C2 phage killed approximately 95% of *E. coli* E1 biofilm cells (Figure 4a). The colony-forming unit was also determined, and we observed a significant reduction in the colonies of *E. coli* E1 in the combinatorial treatment of vB_EcoM_C2 and neem extract, as compared to the sole treatment (4b). As a result, the attributed finding is that the combined treatment is far more effective than the sole treatment of phage and neem extract for the control of *E. coli* E1 biofilm.

Explanation of the mechanism based on the result

Mechanism1: Neem extract is responsible for the killing of bacteria by targeting the cell membrane and inhibiting extracellular polysaccharides (EPS), however, the exact target mechanism is still not clear. In the current study, the targeting of cells (*E. coli* E1) with dual antimicrobial neem extract and bacteriophage reduces the chances of survival of targeted bacteria.

Mechanism2: Since bacteria are continuously evolving, they have evolved several resistance mechanisms against bacteriophages, such as the CRISPR-cas system, restriction of phage DNA entry, and damage of phage DNA. Therefore, *E. coli* cells acquire resistance against the applied phage, then the immunity of bacteria gets compromised, and targeting these immune compromised cells by neem extract might enhance the killing rate at the same time. In a biological system, genetic trade-off mechanisms generally come into play when cells become resistant to one antimicrobial; in that case, they become more susceptible to other antimicrobials.

Mechanism3: Bacteria are commonly used to live in the biofilm form. A biofilm is a complex community of microbes in which they are embedded in a thick EPS matrix. Neem extract has the ability to inhibit EPS production; when the EPS is disrupted, the biofilm loses its integrity, and the bacterial cells disperse. The dispersed cells are more susceptible to bacteriophages.

Conclusion

The pathogenic multiple drug-resistant bacteria *E. coli* E1 was isolated from drinking water, and the combinatorial treatment approach (bacteriophage and neem extract) significantly inhibited the growth of *E. coli* E1 biofilm cells as compared to sole or single treatment (bacteriophage or neem extract). As per our results, neem extract might hamper the biofilm formation of *E. coli* E1 by

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inhibiting the EPS production and free planktonic (free cells of *E. coli*) cells might be more susceptible to bacteriophage. Hence, the survival rate of *E. coli* E1 was minimized when we applied neem extract in combination with bacteriophage. The current study indicates that the combinatorial treatment approach may be an alternative way to treat multiple drug-resistant bacteria effectively, and it opens a new venue for the clinician to control multiple drug-resistant bacterial pathogens. The phage and neem extract remedy may be helpful to the clinician in controlling biofilm forming multiple drug resistant *E. coli*.

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Accession no.: Isolated bacteria sequence was submitted to the gene bank and accession no. is MW590616.

Author contributions

SSS: Conceptualization, Manuscript writing, and data analysis; SR and SPS: sample collection and data analysis.

Supplemental Material

Supplemental material for this article is available online.

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