

## The use of cinnamon oil as antibacterial agent to eliminate some antibiotic-resistant bacteria isolated from water sources

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Extended-spectrum  $\beta$ -lactamases (ESBL) are enzymes produced by Gram-negative microorganisms, which may be resistant to commonly used antibiotics. The purpose of this research was to estimate the bactericidal effects of cinnamon oil on ESBL-producing bacteria. In this study, 227 water samples were collected from wells in Hafr Al-Batin, Saudi Arabia. The samples were cultured on a cystine lactose electrolyte-deficient (CLED) medium. A MicroScan system was used to identify bacteria and also for antimicrobial susceptibility test. Activity of crude cinnamon oil and its fractions were detected by determining the minimum inhibitory concentration (MIC) against the ESBL-producing bacteria. Morphological changes of the treated bacteria were observed and oil compounds were investigated. The culture was positive on the CLED medium in 170 out of 227 water samples. In 170 CLED-positive isolates, *E. coli* was the most common organism, followed by *K. pneumoniae*. The results showed that 100% of *K. pneumoniae* isolates were completely resistant to ampicillin (100%), then by mezlocillin (92.5%), cefazolin, and cefuroxime (77.5%). Also, 86.9% of *E. coli* isolates were the most resistant to ampicillin, followed by mezlocillin (83%). 82% of *K. pneumoniae* and 89% of *E. coli* isolates were confirmed by phenotypic confirmatory disc diffusion test (PCDDT) as ESBL-producers. The cinnamon oil activity was only concentrated in the oxygenated fraction. The MICs of the oxygenated fraction were 80 and 20  $\mu\text{l/mL}$  at  $10^5$  CFU of ESBL-producing *E. coli* and *K. pneumoniae*, respectively. This study indicated the antibacterial effects of cinnamon essential oil to eliminate some antibiotic-resistant bacteria from water.

**Keywords:** water, *Escherichia coli*, *Klebsiella pneumoniae*, antibiotic resistance, essential oil

### 1 Introduction

Some microorganisms, including *Escherichia coli* and *Klebsiella* species, have the ability to produce large amounts of extended-spectrum  $\beta$ -lactamases (ESBLs) (Al Yousef et al., 2016). These enzymes can cause multidrug resistance and lead to urinary tract infections, which are difficult to treat (Zorc et al., 2005). Members of the Enterobacteriaceae family, especially *E. coli* and *Klebsiella* species harboring ESBLs, have been recognized since the 1980's as the main causes of hospital-acquired infections.

Many bacteria, which are responsible for urinary tract infections and loose stools, have the capacity to be ESBL producers. These bacteria include *E. coli*, *Salmonella*, *Shigella*, and *Vibrio cholerae* (Doi et al., 2007). ESBLs and AmpC  $\beta$ -lactamases are a cause of increasing clinical

concern. ESBLs are commonly produced by *Klebsiella* species and other Gram-negative bacteria. Clavulanate enzymes are known to break down penicillin, extended-spectrum cephalosporins, and aztreonam (Bradford, 2001). For the evaluation of ESBLs, susceptibility to cefotaxime, ceftazidime, ceftriaxone, and cefpodoxime was examined, followed by phenotypic corroborative testing (Moland et al., 2002).

ESBLs are competent enzymes, responsible for pathogen resistance to penicillin and first-, second-, and third-generation cephalosporins. They are not active against cephamycin but are rather susceptible to clavulanic acid (Rosenthal et al., 2010). Because of antibiotic overuse, the antimicrobial resistance of bacteria has increased around the world (Gaspari et al., 2005; Stürenburg and Mack,

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2003). Bacteria responsible for urinary tract infections (UTIs) show resistance to conventional medications. The effectiveness of ESBL screening may alter according to the type of antimicrobial agent (Mackenzie et al., 2002), as indicated by the Clinical and Laboratory Standards Institute (CLSI) M100-S11 guidelines (Patel et al., 2001).

Antibacterial resistance monitoring is essential for identifying problems and choosing an appropriate antimicrobial drug for therapy. Therefore, the development of new active antimicrobials, with the fewest side effects, is essential (Reddy and Yang, 2015). Different studies have demonstrated the positive effect of oils on different types of microorganisms, such as *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli*, *Bacillus cereus*, and *Salmonella typhimurium* (Burt, 2004; Brenes and Roura, 2010; Pesavento et al., 2015). Evidence shows that phenolic mixtures are the most dynamic against these bacteria. Consequently, cinnamon, thyme, and rosemary oils are considered as the most successful agents against microorganisms (Dorman et al., 2000; Ojagh et al., 2010). The antimicrobial effects of cinnamon oil on different microbial agents, such as *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *S. aureus*, *Salmonella* species, and *Vibrio parahaemolyticus*, have been reported in the literature (Chang et al., 2008).

This study aimed to define the current trend of ESBL production in bacterial isolates collected from the water. Also, the antimicrobial activity of cinnamon essential oil was examined alone and in combination with antibiotics against pathogenic microorganisms in water.

## 2 Material and methods

### 2.1 Study samples

This study was performed at the Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Hafr Al-Batin University (Saudi Arabia) from October 2016 to October 2019.

### 2.2 Samples collection and processing

In Hafr Al-Batin, Saudi Arabia, approximately 100% of water supply originates from groundwater sources. In this city, water is transferred from a vehicle to the client's house and is stored in wells, which may be situated next to sewage tanks. Under aseptic conditions, 250 mL sterile bottles were used to collect water for the tests. The samples were transferred to the research center on ice and examined for four hours. A total of 227 samples were collected from the wells. Water samples were centrifuged at 6,000 rpm for 12 minutes at 4 °C. The pellets were streaked on the CLED medium agar and incubated at 37 °C for 48 hours. Out of 227 samples collected, the

culture of only 170 isolates was positive on the CLED medium.

### 2.3 MicroScan analysis

The CLED-positive isolates (170) were subjected to MicroScan analysis. A MicroScan WalkAway-96 SI system was used for detecting antibiotic sensitivity. Antimicrobial susceptibility testing was performed using Negative BP/Combo 30-B1017-306E combination panels. The methodology was performed according to the manufacturer's instructions. The integrated LabPro version 1.12, which incorporates the Alert expert system, uses growth in the presence of cefpodoxime (4 µg mL<sup>-1</sup>) and ceftazidime (1 µg mL<sup>-1</sup>), i.e., at concentrations proposed by the National Committee for Clinical Laboratory Standards (NCCLS) for ESBL screening (Jones, 1986) as the primary indicators of potential ESBL production. The minimum inhibitory concentrations (MICs) of ceftriaxone and cefotaxime were determined according to the CLSI breakpoints (Patel et al., 2001). It should be noted that screening with this system is restricted to *E. coli*, *K. pneumoniae*, and *K. oxytoca* (i.e., primary species in the CLSI guidelines).

### 2.4 ESBL confirmatory tests

Out of 170 CLED-positive isolates, 104 were found positive in the MicroScan analysis. The collected isolates were subjected to ESBL confirmatory tests, which are described below.

#### 2.4.1 Double disc synergy test (DDST)

The colonies were collected and recultured in peptone water at 37 °C for 2–6 hours. The 0.5 McFarland standard was also set, and cultures were prepared on Mueller-Hinton agar. Augmentin (20/10 µg) was placed in the middle of the dish. The cefotaxime (30 µg) and ceftazidime (30 µg) discs were placed at a 15 mm distance on two sides of the augmentin disc, and the dish was grown at 37 °C. The ESBL production was determined according to a study by Ramesh et al. in 2019 (Ramesh et al., 2019).

#### 2.4.2 Phenotypic confirmatory disc diffusion test (PCDDT)

ESBL production was confirmed in potential ESBL-producing isolates by phenotypic tests. The culture of microbes was prepared, and third-generation cephalosporin, ceftazidime (30 µg), and ceftazidime + clavulanic acid (30 µg + 10 µg) discs were placed at 25 mm distances; the dish was kept at 37 °C. ESBL production was confirmed according to a study by Lal et al. in 2007 (Lal et al., 2007).

## 2.5 Essential oil

### 2.5.1 Extraction and fractionation

The cinnamon bark was chopped into small pieces and distilled using a Clevenger-type apparatus. Oil was extracted, dried in sodium sulfate anhydrous, and stored at 4 °C in sealed dark bottles until further bioassays and investigations. Oil was fractionated into oxygen and hydrocarbon fractions by flash chromatography on silica gel using a glass column. The fractions were assayed against each test organism.

### 2.5.2 Gas chromatography (GC) and GC-mass spectroscopy (GC-MS)

The oxygenated and hydrocarbon fractions of cinnamon oil were analyzed via GC, using a DB-S capillary column. The oven temperature was set at 50 °C. The GC-MS conditions were similar to GC. The electron ionization was set at 70 eV, the particle source temperature was set at 275 °C, and the output rate was 29–400 atomic mass units sec<sup>-1</sup>.

### 2.5.3 Identification of cinnamon oil components

Authentic components were injected, and comparisons were made in terms of the retention time, Kovats index, and mass spectra. The latter parameter was compared with the mass spectra of the National Bureau of Standards (NBS) library and spectra.

### 2.5.4 Antibacterial activity of cinnamon essential oil

The antibacterial activity of oxygenated and hydrocarbon fractions was examined in air space. The preliminary examination of the activity of oxygenated and hydrocarbon fractions against ESBL-resistant *E. coli* and *K. pneumoniae* isolates was carried out. The oil concentration ranged from 1 to 16 µL per 0.4l L of air space, based on the overturned Petri dish method. The paper discs (5 mm diameter) were impregnated with oil concentrations and placed on a Petri dish. The plate dimensions were 140 × 23 mm, providing 0.4l mL of air space after pouring the medium. Next, a loop from a 24-hour-old culture of tested bacteria was streaked on the CLED medium. The plates were sealed with parafilm and grown at 36 °C for 24 hours. Finally, colony formation was observed. For the controls, distilled water was used rather than oil.

### 2.5.5 MIC of oxygenated fraction

MIC was expressed as the lowest concentration of oil, preventing the visible growth of microbial agents. It was determined using the lysogeny broth (Sigma, St. Louis, MO, USA). Bacterial concentration was determined

based on the McFarland standards. The bacterial isolates were two-fold dilutions of the oxygenated fraction. Cell viability was examined after 24 hours of incubation, using a colony-forming assay on a nutrient agar (Kohanski et al., 2007).

### 2.6 Scanning electron microscopy (SEM)

Changes in the treated bacterial cells were observed using SEM, according to the method proposed by Diao et al. (Diao et al., 2013). Bacterial cells were incubated in nutrient broth (NB) at 37 °C for ten hours, and then, the suspensions were added to the MIC of the oxygenated fraction (80 µl mL<sup>-1</sup> and 20 µl mL<sup>-1</sup> for *E. coli* and *K. pneumoniae*, respectively). The suspension was incubated at 37 °C for four hours; afterward, the suspension was centrifuged at 5,000 rpm for ten minutes. The cells were washed twice with 0.1 M phosphate-buffered saline (PBS; pH = 7.4) and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M PBS at 4 °C. Next, the cells were gradually dried using 30%, 70%, 90%, and 100% ethanol, and ethanol was supplemented with tert-butyl alcohol. The cells were gold-coated via a cathodic coating, and the morphology of bacterial cells was determined using SEM (INSPECT S50) at the College of Sciences of Hafr Al-Batin University.

## 3 Results and discussion

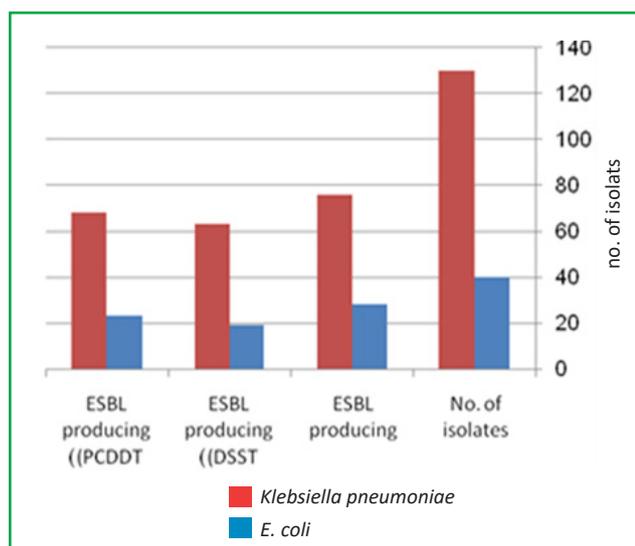
Out of 227 water samples, 170 were culture-positive on the CLED medium. The most familiar microorganism was *E. coli* ( $n = 130$ , 76.5%), followed by *K. pneumoniae* ( $n = 40$ , 23.5%). The antibiotic susceptibility test indicated the complete resistance of *K. pneumoniae* to ampicillin (100%), followed by mezlocillin (92.5%), cefazolin (77.5%), and cefuroxime (77.5%), respectively; the test indicated the highest sensitivity to imipenem (85%). *E. coli* showed the highest resistance to ampicillin (86.9%), followed by mezlocillin (83%) and amikacin (77.7%), respectively. The test showed the highest sensitivity to fosfomicin (90.7%), imipenem (90.7%), and piperacillin/tazobactam (86.92%), respectively (Table 1).

A total of 170 isolates were screened for ESBL production, using the MicroScan WalkAway-96 SI system, according to the CLSI guidelines. The results showed that 104 (61.18%) isolates were positive for ESBL production. Among 104 ESBL-positive isolates, 76 were *E. coli*, and 28 were *K. pneumoniae* (Table 1).

The ESBL-positive isolates were selected for the corroborative trial of ESBL. Two procedures, i.e., DDST and PCDDT, were performed to confirm ESBL production. Based on the results, out of 28 *K. pneumoniae* isolates, which were ESBL-positive in the MicroScan screening, 19 were positive on DDST (67%), and 23 (82%) were

**Table 1** Antibiogram of isolated *K. pneumoniae* and *E. coli* by Microscan.

Antibiotic	<i>Klebsiella pneumoniae</i>		<i>Escherichia coli</i>	
	no. of isolates and percentage (%)			
	resistant	sensitive	sensitive	resistant
Amikacin	9 (22.5)	31 (77.5)	29 (22.3)	101 (77.7)
Amoxicillin/Clavulanic	22 (55)	18 (45.0)	68 (52.3)	62 (47.7)
Ampicillin	40 (100)	0 (0.00)	17 (13.08)	113 (86.92)
Cefazolin	31 (77.5)	9 (22.5)	68 (52.3)	62 (47.7)
Cefepime	30 (75)	10 (25.0)	74 (57.0)	56 (43.07)
Cefotaxime	28 (70.0)	12 (30.0)	74 (57.0)	56 (43.07)
Cefoxitin	19 (47.5)	21 (52.5)	85 (65.85)	45 (34.61)
Cefuroxime	31 (77.5)	9 (22.5)	68 (52.3)	62 (47.70)
Ciprofloxacin	24 (60.0)	16 (40.0)	45 (34.46)	85 (65.44)
Ertapenem	9 (22.5)	31 (77.5)	107 (82.30)	23 (17.70)
Fosfomycin	15 (37.5)	25 (62.5)	118 (90.7)	12 (9.3)
Gentamicin	18 (45)	22 (55.0)	63 (48.46)	67 (51.54)
Imipenem	6 (15.0)	34 (85.0)	118 (90.7)	12 (9.3)
Levofloxacin	15 (37.5)	25 (62.5)	62 (47.7)	68 (52.30)
Mezlocillin	37 (92.5)	13 (7.50)	22 (17.0)	108 (83.00)
Pipracillin/Tazobactam	9 (22.5)	31 (77.5)	113 (86.92)	17 (13.08)
Tetracycline	9 (22.5)	31 (77.5)	44 (33.84)	86 (66.16)
Tobramycin	25 (62.5)	15 (37.5)	51 (39.23)	79 (60.77)
Trimethoprim/Sulfamethoxazole	25 (62.5)	15 (37.5)	44 (33.85)	86 (66.15)



**Figure 1** ESBL Pattern of *Klebsiella pneumoniae* and *E. coli* isolates

positive on PCDDT. On the other hand, 76 *E. coli* isolates were ESBL-positive in the MicroScan screening, 63 (83%) were positive on DDST, and 68 (89%) were positive on PCDDT (Figure 1).

Analysis of cinnamon oil indicated that cinnamaldehyde and eugenol were the major components of the oxygenated fraction (57.17% and 15.64% relative to oil, respectively). Camphene was the major component of the hydrocarbon fraction, while cinnamaldehyde was the major component of the oxygenated fraction (Table 2). Nineteen compounds comprising the oxygenated fraction were identified via GC-MS and literature review. Generally, cinnamon oil is characterized by high levels of oxygen-containing monoterpenes, including cinnamaldehyde, which is the main component of cinnamon essential oil.

The susceptibility of ESBL-producing bacteria to the volatiles of oxygenated and hydrocarbon fractions of cinnamon oil was examined using the inverted Petri dish method, as shown in Table 3. Bacterial growth suppression was only observed in the oxygenated fraction. *K. pneumoniae* showed high sensitivity to the

**Table 2** Chemical composition of the essential oil from cinnamon analyzed by GC-MS.

Oxygenated fraction			Hydrocarbon fraction		
RT	Component	%Area	RT	Component	%Area
9.42	fenchone	0.02	10.38	$\alpha$ -pinene	0.64
10.11	camphor	3.06	14.99	camphene	83.79
12.50	linalool	0.1	17.36	not know	0.21
13.91	methyl chavicol	4.33	21.81	sabinene	8.37
15.08	geranyl acetate	12.69	24.41	myrcene	0.85
19.77	phenylethyl acetate	0.24	27.71	$\alpha$ -phellandrene	0.08
21.25	nerol	0.18	29.67	$\alpha$ -terpinene	5.28
23.00	cinnamaldehyde	57.17	32.19	limonene	0.03
23.77	geraniol	0.22	36.03	not know	0.07
25.47	eugenol	15.64	37.18	benzyl $\alpha$ -toluate	0.43
30.36	2-phenylpropyl acetate	1.35	39.43	$\gamma$ -terpinene	0.10
30.61	not know	1.04	42.64	$\beta$ -cymene	0.05
35.37	caryophyllene oxide	0.02	44.17	terpinolene	0.10
36.14	methyleugenol	0.13	45.64	$\alpha$ -humulene	0.07
37.38	benzyl $\alpha$ -toluate	1.77	46.72	$\beta$ -selanene	2.3
39.57	eugenol	0.02	47.13	$\gamma$ -cttadenene	0.5
40.34	benzyl hydrocinnamic acid	0.05			
41.99	unknown	0.01			
44.03	benzyl cinnamate	0.86			
45.46	not know	0.03			
50.19	benzyl ether	0.29			
51.89	benzylthioacetic acid, benzyl ester	0.78			

**Table 3** Activity of oxygenated and hydrocarbon fraction of cinnamon oil against ESBL-producing bacteria using inverted Petri dish technique.

Bacteria	Oxygenated fraction ( $\mu\text{l } 0.4 \text{ l}^{-1}$ air space)					Hydrocarbon fraction ( $\mu\text{l } 0.4 \text{ l}^{-1}$ air space)				
	1	2	4	8	16	1	2	4	8	16
<i>E. coli</i>	+	+	+	-	-	+	+	+	+	+
<i>K. pneumoniae</i>	+	-	-	-	-	+	+	+	+	+

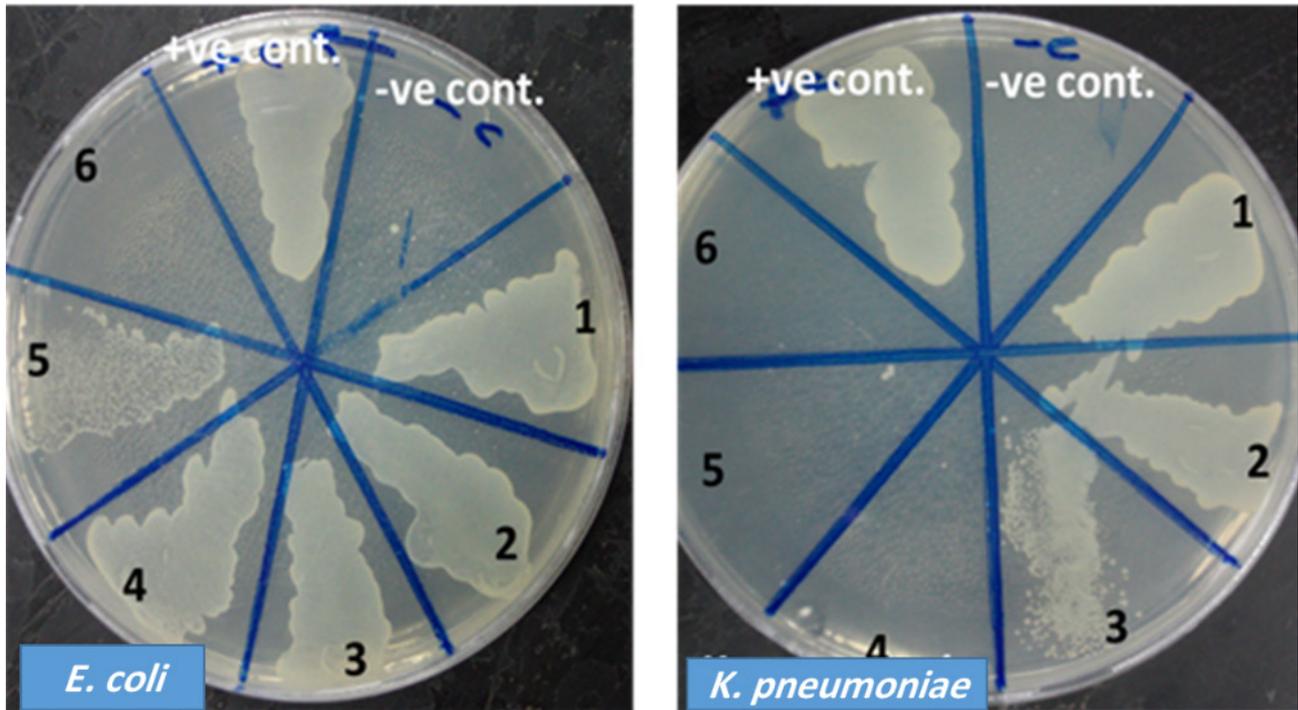
(+) meaning bacterial growth, (-) meaning no bacterial growth

oxygenated fraction. Its growth was suppressed at a concentration of  $2 \mu\text{l } 0.4 \text{ L}^{-1}$  air space. *E. coli* growth was suppressed at a concentration of  $8 \mu\text{l } 0.4 \text{ L}^{-1}$  air space (Table 3).

The ESBL-producing *E. coli* and *K. pneumoniae* isolates were exposed to two-fold serial dilutions of the oxygenated fraction for 24 hours. The oxygenated fraction influenced the cellular viability of ESBL-producing bacteria in a dose-dependent manner. The bacterial cell viability was monitored to determine the bactericidal

effect of different concentrations of the oxygenated fraction on *E. coli* and *K. pneumoniae*. The bacterial cell viability was also estimated using a colony-forming assay in a nutrient agar. The *E. coli* and *K. pneumoniae* isolates were suppressed at concentrations above 80 and  $20 \mu\text{l mL}^{-1}$  at  $10^5$  CFU, respectively, where no bacterial growth was seen in the agar plates at the evaluated oil concentrations (Figure 2).

The study of the morphological changes of microorganisms is essential for distinguishing the

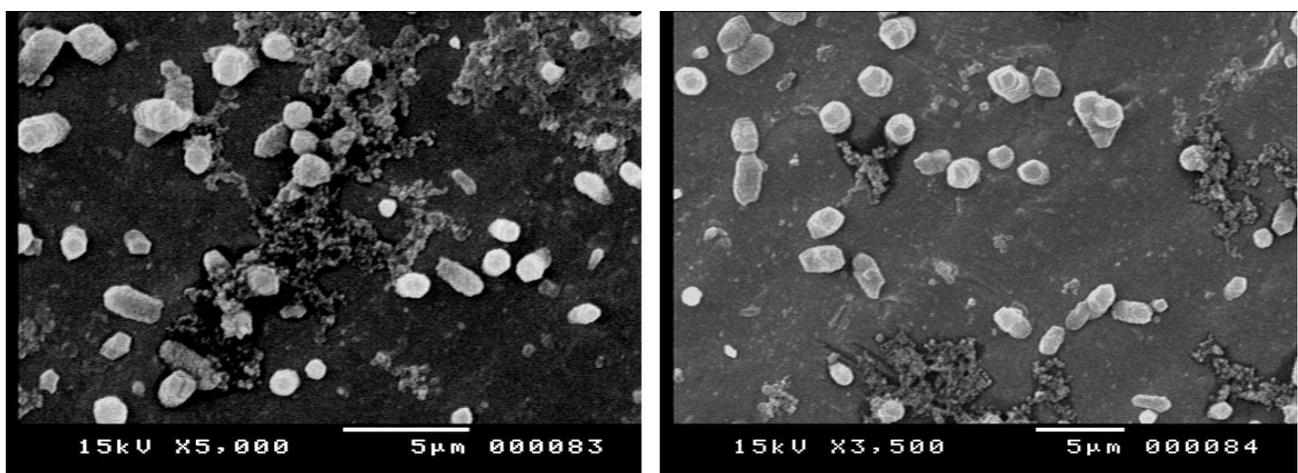


**Figure 2** Colony-Forming Capacity Assay  
1 – growth at concentration of 2.5  $\mu\text{L mL}^{-1}$  of oxy. Fraction, 2 – growth at 5  $\mu\text{L mL}^{-1}$ , 3 – growth at concentration of 10  $\mu\text{L mL}^{-1}$ , 4 – growth at 20  $\mu\text{g mL}^{-1}$ , 5 – growth at concentration of 40  $\mu\text{L mL}^{-1}$ , 6 – growth at concentration of 80  $\mu\text{L mL}^{-1}$ , compared with positive and negative controls

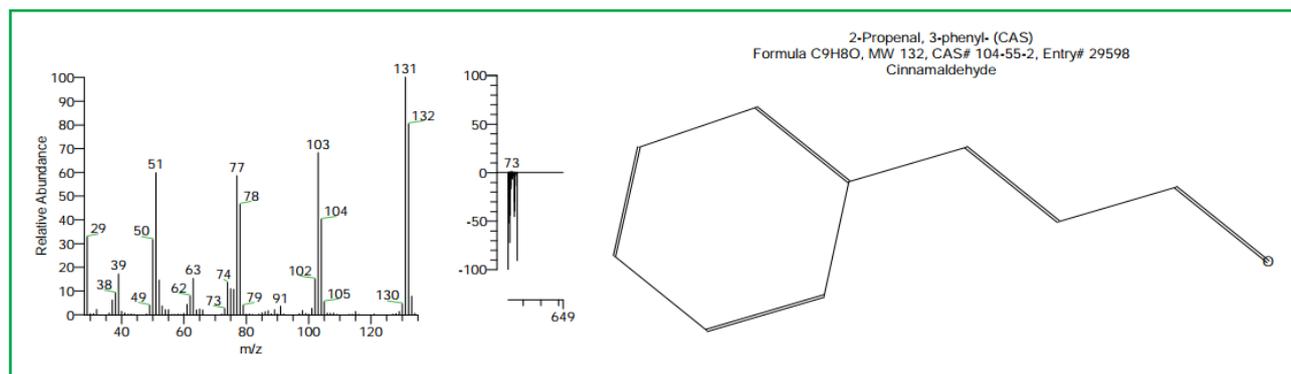
antibacterial properties of cinnamon oil. SEM was used to explore the surface morphology of *E. coli* and *K. pneumoniae* after cinnamon oil treatment in the NB medium (Figure 3). The treated bacterial cells were significantly damaged, as shown in TEM images.

The current study clearly showed that *E. coli* and *K. pneumoniae* isolates from water were resistant to third-generation cephalosporins and sensitive to carbapenems. The findings also revealed an alarming increase in the prevalence of resistance to commonly used antibiotics.

Similar results were reported by Shakibaie et al., 2014 who isolated *E. coli* from UTI children in the pediatric unit of a medical clinic in Kerman, Iran. Also, they found that 100% of isolates were sensitive to carbapenems, and 94.4% were sensitive to amikacin; all uropathogenic *E. coli* isolates were resistant to ampicillin (100%). Likewise, in our study, *K. pneumoniae* showed complete resistance to ampicillin (100%), followed by mezlocillin (92.5%). On the other hand, it showed the highest sensitivity to both imipenem and fosfomycin (90.7%). Consistent results were reported by Adeyemi et al., 2014 who found an



**Figure 3** SEM image of *E. coli* (right) and *K. pneumoniae* (left) after oxygenated fraction of cinnamon oil treatment



**Figure 4** GC-MS of cinnamylaldehyde

increase in *E. coli* and *Klebsiella* spp. resistance to most antibiotics.

A standard procedure is necessary for the identification of ESBL-producing microorganisms, based on standard identification methods to control the spread of diseases and propose appropriate treatment plans. The current study showed that PCDDT was more sensitive than DDST in identifying ESBL. DDST identified 67% of *K. pneumoniae* isolates and 83% of *E. coli* isolates, whereas PCDDT detected 82% of ESBL-producing *K. pneumoniae* isolates and 89% of *E. coli* isolates. According to some studies, the percentage of ESBL-producing bacteria ranges from 28% to 84% in India (Narayanaswamy and Mallika, 2011). The percentage of these bacteria was the lowest in Maharashtra, India, as reported by Rodrigues et al. (Rodrigues et al., 2004). The high prevalence of ESBL-producing *K. pneumoniae* has also been reported by other researchers (Lal et al., 2007; Ramesh et al., 2019).

Aromatic volatile components of plants exhibit higher antimicrobial activity than non-aromatic volatile oils (Shaaban et al., 2012). Our results are in line with previous studies, which indicated the antimicrobial activity of cinnamon oil (Raeisi et al., 2015; Lin et al., 2017); however, the cell membrane may be injured after treatment. These findings showed that cinnamon oil could destroy the bacterial cell membrane, causing cell lysis and death. Cinnamaldehyde was found to be the main component of cinnamon essential oil (Figure 4); this result may represent the mechanism of action of the oxygenated fraction.

#### 4 Conclusions

The results of the present study showed that cinnamon oil and its components could be used as antibacterial agents to prevent bacteria-induced water contamination. Moreover, *in vivo* studies regarding the mechanism of the antibacterial activity of cinnamon oil should be performed.

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