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ORIGINAL ARTICLE

Cinnamaldehyde: a compound with antimicrobial and synergistic activity against ESBL-producing quinolone-resistant pathogenic *Enterobacteriaceae*

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Abstract

Usage of cephalosporin and quinolone antibiotics has aggravated the development of extended-spectrum beta-lactamase (ESBL)–producing quinolone-resistant (QR) pathogenic *Enterobacteriaceae.* The present study aims to determine antimicrobial activity of cinnamaldehyde alone or in combination with cefotaxime/ciprofloxacin to reverse the drug resistance and evaluations of efficacy, and possible molecular mechanism of action of the combination was also evaluated using in vitro assays. Broth microdilution assay was used to determine minimum inhibitory concentrations (MICs) of cinnamaldehyde and antibiotics against ESBL-QR *Enterobacteriaceae*. Synergistic effect and dynamic interaction with antibiotics were further examined by checkerboard assay, isobologram analysis, and time-kill assay, respectively. Cellular morphology of bacteria was viewed with scanning electron microscopy (SEM). Effects of cinnamaldehyde and its combination on the expression of gene encoding—porins (*ompC*, *ompF*, *ompK35*, and *ompK36*), efflux pump genes (*acrB–E. coli*, *acrB–K. pneumoniae*), and antibiotic-resistant genes (*blaTEM*, *blaSHV*, *blaCTXM*, and *QnrB*) were evaluated using real-time quantitative PCR (RT-qPCR). Majority of the *E. coli* (32.1%) and *K. pneumoniae (*24.2%) isolates demonstrated MIC of cinnamaldehyde at 7.34 μg/mL and 0.91 g/mL, respectively. Synergism between cinnamaldehyde and cefotaxime was noted among 75% *E. coli a*nd 60.6% *K. pneumoniae.* Similarly, synergism with ciprofloxacin was observed among 39.6% and 42.4% of the bacteria, respectively. Thus, cinnamaldehyde reduced MIC of cefotaxime and ciprofloxacin 2–1024-fold with bactericidal and/synergistic effect after 24 h. Cinnamaldehyde and its combination altered gene expression by ~1.6 to ~400-fold. Distorted bacterial cell structures were visible after treatment with cinnamaldehyde and/or with cefotaxime/ciprofloxacin. The results indicated the potential efficacy and mode of action of cinnamaldehyde alone and in combination with antibiotics against pathogenic ESBL-QR bacteria.

Keywords Cinnamaldehyde · ESBL · Quinolone · Synergy · Scanning electron microscopy · Real-time quantitative PCR

Introduction

Indiscriminate and irrational usage of cephalosporin and quinolone antibiotics has aggravated the development of extended-spectrum beta-lactamase (ESBL)–producing quinolone-resistant (QR) pathogenic *Enterobacteriaceae*, thereby reducing efficacy of cephalosporins and quinolone drugs against these bacteria [1–3]. ESBL production with quinolone resistance development among *Enterobacteriaceae* results from production of beta-lactamases (*TEM*, *SHV*, and *CTX-M*), expression of QNR genes, enhanced efflux pump expression (*AcrAB-TolC*), and alteration of outer membrane permeability (*OmpF/OmpC*: *Escherichia coli* and *Ompk35/Ompk36*: *Klebsiella pneumoniae*, respectively) [4, 5]. Thus, development of an alternative drug line to treat and control ESBL-QR pathogenic bacteria is urgently needed. Plant bioactive compounds with intrinsic antimicrobial properties may offer a plethora of interesting possibilities to combat antibiotic resistance [6]. The use of plant bioactive compounds in combination with conventional antibiotics has been proposed to be an effective method to control multidrug resistance as this combination targets multiple facets of infectious agents. Cinnamaldehyde demonstrated synergistic interaction with various antibiotics against Gram-positive and Gram-negative bacteria [7–11].

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However, antibacterial activity of cinnamaldehyde along with its combinatorial effect with traditional antibiotics has not yet been tested against ESBL-QR *Enterobacteriaceae*. The present study aims to analyze antibacterial and synergistic activity of cinnamaldehyde against pathogenic ESBL-QR *Enterobacteriaceae* and evaluate their effect through several antibacterial, microscopic, and gene-expressional analysis to find out its therapeutic potential in antibacterial applications.

Materials and method

Bacterial sample collection

After obtaining institutional ethical committee approval, sixty-one ESBL-QR clinical isolates (*E. coli* ($n = 28$); *K. pneumoniae* ($n = 33$)) were collected from unrelated patient's visiting Calcutta School of Tropical Medicine (ref. no. CREC-STM/53 dated 23/09/2011). All ESBL-QR bacteria demonstrated ESBL property against ceftazidime, cefotaxime, their inhibitor combinations, and quinolone resistance against any three of the following quinolone drugs: nalidixic acid, ciprofloxacin, and levofloxacin prulifloxacin (Hi-Media Lab Ltd., India).

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) of cinnamaldehyde (CIN) (HiMedia Lab Ltd., India), cefotaxime (CTX), and ciprofloxacin (CIP) against ESBL-QR bacteria were determined by microbroth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute [12]. MIC of each compound against a maximum number of ESBL-QR *Enterobacteriaceae* was considered as MIC (mode-MIC value) of that compound, and those concentrations (MIC-CIN, MIC-CTX, and MIC-CIP) were selected for downstream studies.

Determination of synergism between cinnamaldehyde and antibiotics

Combinatorial effects of CIN with antibiotics were determined by the checkerboard method [13]. Twofold serial dilutions of each compound were prepared to achieve final concentrations of CIN and antibiotics from 0.22 to 7.28 μg/mL and 0.5 to 512 μg/mL, respectively. Synergistic interactions were validated using the CompuSyn software version1.0 to generate isobologram, combination index (CI), and drug-reduction index (DRI) [14].

Time-kill kinetics assay

To determine the dynamic interaction of CIN and antimicrobial agent(s) against ESBL-QR isolates, the time-kill test was performed according to the CLSI guidelines and Zhou et al. [12, 15].

Bacterial membrane integrity assay

Intactness of bacterial cell membrane was measured by fluorescence using Live/Dead BacLight assay kit (Thermo Fisher Scientific, USA) according to manufactures' protocol [16]. Bacterial cells were incubated in the presence of MIC-CIN/CTX/CIP alone and their combinations and visualized under a fluorescence microscope (LEICA DM2000, India).

Bacterial morphology study

Morphological changes of bacterial cells grown for 6 h at 37 °C in MHB supplemented with or without $^1/_2$-MIC-CIN/CTX/CIP alone and their combinations were visualized using scanning electron microscope (SEM) (ZEISS EVO-MA 10, Denmark) following standard protocol [17].

Transcriptional expression profiles of antibiotic-resistant genes, efflux pump gene, and porins

Quantitative RT-PCR was performed to determine changes in expression of antibiotic-resistant genes (*blaTEM*, *blaSHV*, *blaCTXM*, and *QnrB*), efflux pump (*acrB–E. coli*, *acrB–K. pneumoniae*), and porins (*ompC*, *ompF*, *ompK35*, and *ompK36*) in the presence and absence (untreated control) of $^1/_2$-MIC CIN alone and combined with CTX/CIP [15]. Quantification of target genes was performed on ABI Prism 7500 using Power SYBR Green PCR MasterMix (Applied Biosystems, USA). Oligonucleotides used in this study were mentioned in online resource 1. Relative level of target gene expression compared with 16S ribosomal RNA (internal control) was determined by calculating $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Time-kill curves and RT-PCR data were expressed as mean ± standard error. Values of the treated groups were statistically compared with those of untreated control group by Student's *t* test and one-way ANOVA performed with Dunnett's HSD post hoc comparison. For CIN- and CTX/CIP-treated group comparison, one-way ANOVA with Tukey's HSD post hoc was performed. $P < 0.01$ and $P < 0.001$ were considered statistically significant and highly significant. Statistical analysis was carried out using GraphPad prism.

Results and discussion

Indiscriminate use of beta-lactam and quinolone antibiotics often results in the development of resistance against these antibiotics. Therefore, investigations of a novel antibacterial candidate to overcome such

resistance would be intriguing. In the present study, efficacy of cinnamaldehyde as candidate molecule for the treatment of ESBL-producing and quinolone-resistant (ESBL-QR) pathogenic bacteria was evaluated.

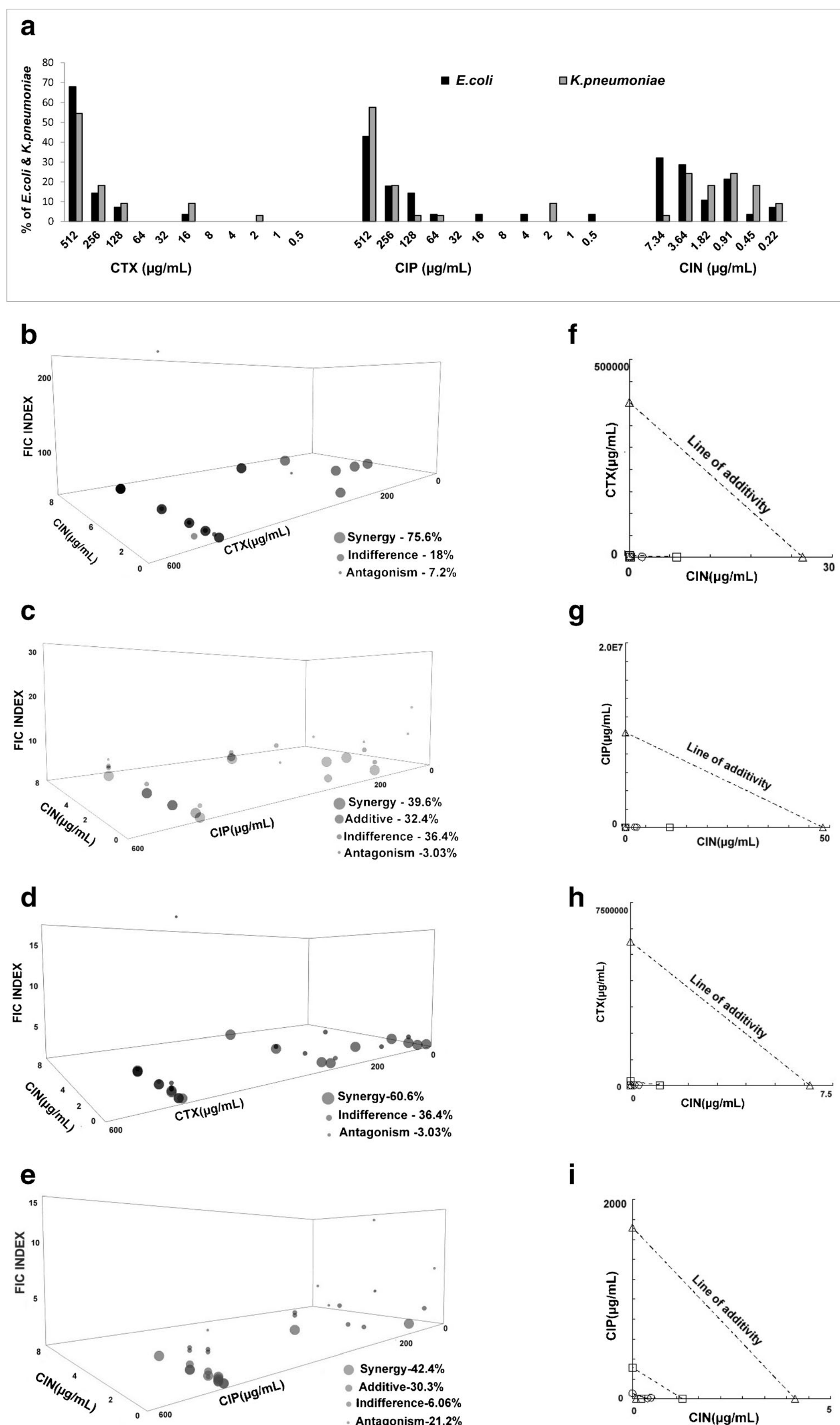

Fig. 1 Antibacterial activity and synergy determination of cinnamaldehyde with antibiotics. **a** Distribution of MICs of cefotaxime (CTX), ciprofloxacin (CIP), and cinnamaldehyde (CIN). **b**, **c** Combined effect of cinnamaldehyde and CTX and CIP against ESBL-QR *E. coli*. **d**, **e** Combined effect of cinnamaldehyde and CTX and CIP against ESBL-QR *K. pneumoniae*. **f**, **g** Isobologram of cinnamaldehyde and CTX and CIP against ESBL-QR *E. coli*. **h**, **i** Isobologram of cinnamaldehyde and CTX and CIP against ESBL-QR *K. pneumoniae*

Microdilution assay demonstrated MIC of CTX and CIP at 512 μg/mL among 67.9% and 42.9% of ESBL-QR *E. coli*, whereas CIN exhibited MIC at 7.34 μg/mL among 32.1% isolates. Likewise, traditional antibiotics and CIN showed MIC at 512 μg/mL and 0.91 μg/mL among 54.54%, 57.6%, and 24.2% ESBL-QR *K. pneumoniae*, respectively—indicating greater efficacy of cinnamaldehyde compared with traditional antibiotics against both organisms (Fig. 1a).

Synergistic interaction between CIN and CTX was noticed among 75% (21/28) *E. coli* and 60.6% (20/33) *K. pneumoniae* with FIC-index (*E. coli*, 0.07–0.3; *K. pneumoniae*, 0.07–0.5) (Fig. 1b, d and online resource 2). In the presence of MIC-CIN, MIC-CTX was reduced 4–1024-fold among *E. coli* and 2–1024-fold among *K. pneumoniae*. However, synergism between CIN and CIP (FIC-index *E. coli*, 0.07–0.5; *K. pneumoniae*, 0.1–0.5) was observed among 39.6% (12/28) *E. coli* and 42.4% (14/33) *K. pneumoniae* (Fig. 1c, e). MIC-CIP was reduced 2–512-fold and 2–1024-fold for *E. coli* and *K. pneumoniae*, respectively, in the presence of MIC-CIN. Further, the presence of combination data points below additive lines, CI between 0.01 and 0.23 and DRI > 98-fold confirmed clear synergistic activity of traditional antibiotics with cinnamaldehyde (Fig. 1f–i and online resource 3**)**.

Time-dependent bactericidal effect of MIC-CIN alone and in combination with antibiotics against ESBL-QR strains indicated a decrease in viable bacterial cell count by ≥2log10 CFU/mL within 2–3 h (Fig. 2a–d). In contrast to CIN and antibiotic-alone treatments, combination regimens displayed better bactericidal activities that persist for 24 h.

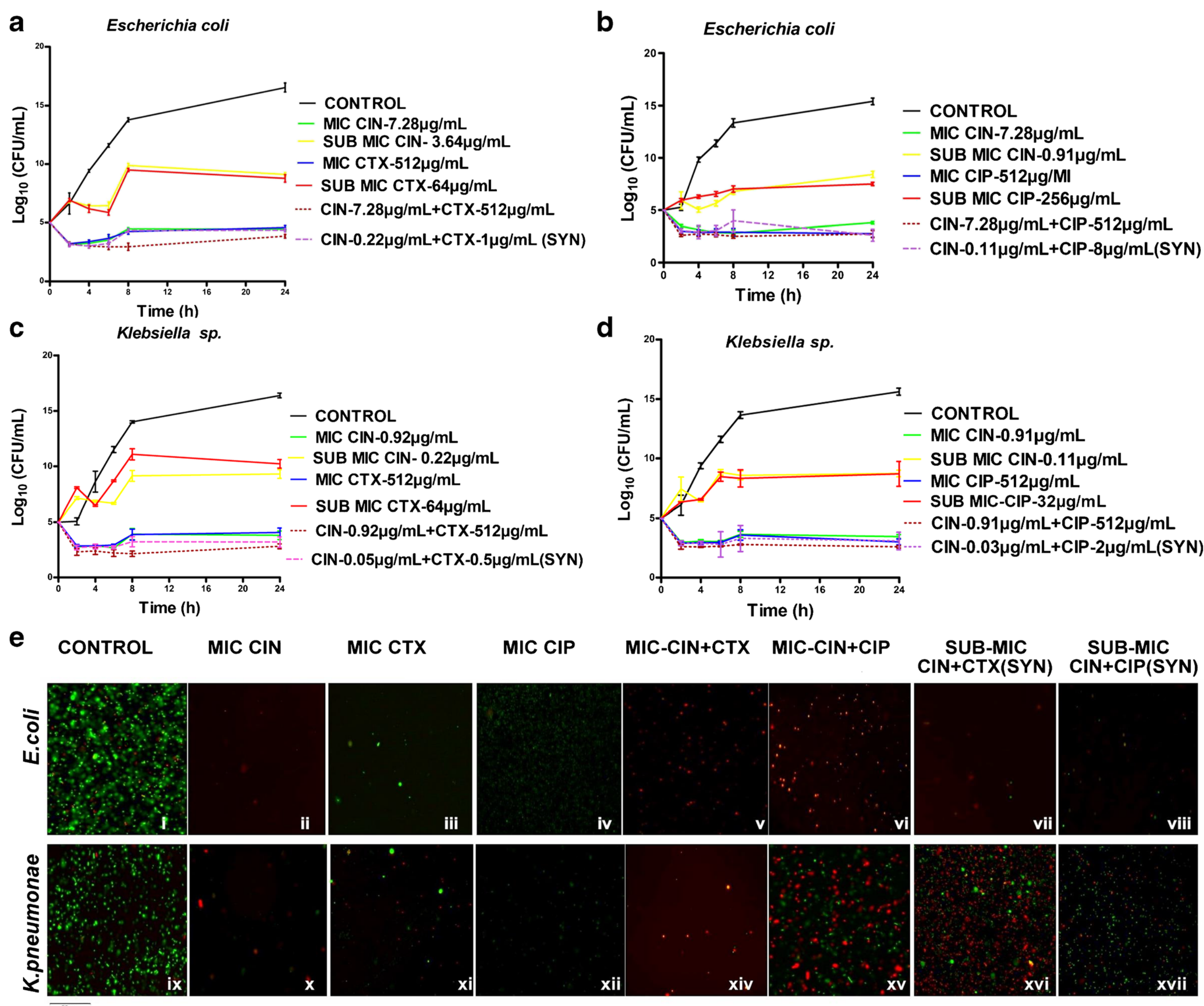

Fig. 2 Time-kill kinetics of cinnamaldehyde with antibiotics alone and its combination on bacterial membrane integrity. Effect of cinnamaldehyde alone and its combination with cefotaxime (CTX) (**a**) and ciprofloxacin (CIP) (**b**) on growth of *E. coli*. Effect of cinnamaldehyde alone and its combination with cefotaxime (CTX) (**c**) and ciprofloxacin (CIP) (**d**) on growth of *K. pneumoniae*. **e** Representative photomicrographs showing cell viability of ESBL-QR *E. coli* (i–viii) and *K. pneumoniae* (ix–xvi). Bar = 50 m

After 8 h of drug treatment, MIC-CIN in combination with MIC-CTX/MIC-CIP had reduced *E. coli* colony count by 0.7- and 0.9-fold compared with MIC-CTX/MIC-CIP alone. At sub-MIC synergistic combination of CIN with CTX/CIP, viable bacterial cell count decreased by 0.5- and 0.4-fold, respectively (Fig. 2a, b).

Similarly, after 8 h of drug treatment, MIC-CIN in combination with MIC-CTX/MIC-CIP reduced *K. pneumoniae* bactericidal activity by 0.6- and 0.8-fold compared with MIC-CTX/MIC-CIP alone (Fig. 2b, d). At sub-MIC synergistic combination of CIN with CTX/CIP, viable bacterial cell count decreased by 0.3- and 0.4-fold.

Fluorescence microscopic analysis of ESBL-QR *E. coli* and *K. pneumoniae* with MIC-CIN alone and in combination with CTX/CIP demonstrated the presence of dead cells (red fluorescence) (Fig. 2e). *E. coli* and *K. pneumoniae* treated with CTX/CIP alone demonstrated the presence of live cells at reduced number compared with untreated control (green fluorescence) (Fig. 2e; iii, iv and xi, xii).

Untreated ESBL-QR *E. coli* and *K. pneumoniae* were visualized as oval- and spherical-shaped cells with very smooth surfaces (Figs. 3a and 4a). However, after treatment with MIC-CTX/CIP alone, *E. coli* exhibited elongated structure with some particulate around their surfaces and *K. pneumoniae* became filamentous with rough surfaces (Figs. 3b, c and 4b, c). This phenomenon has been previously documented as bacterial filamentation as a survival strategy during stressful condition to maintain their morphological plasticity [18]. Some earlier studies reported cefotaxime/ciprofloxacin induced SOS response among ESBL strain, which triggered filamentation [15, 19].

After CIN treatment, while ESBL-QR *E. coli* cell surface became crumpled or grooves appeared on the cell surface with abnormal division of cells, *K. pneumoniae* exhibited cell membrane shrinkage with convoluted surfaces and loosened cell wall indicating initial damage (Figs. 3d and 4d). Such finding was in accordance with earlier studies that demonstrated damage to cell permeability and membrane integrity in pathogenic *Porphyromonas gingivalis* by Cinnamon bark essential oil and cinnamaldehyde [20].

In this study, altered cell surface morphology, shrinkage of cell surfaces, and diminished cytoplasm among ESBL-QR

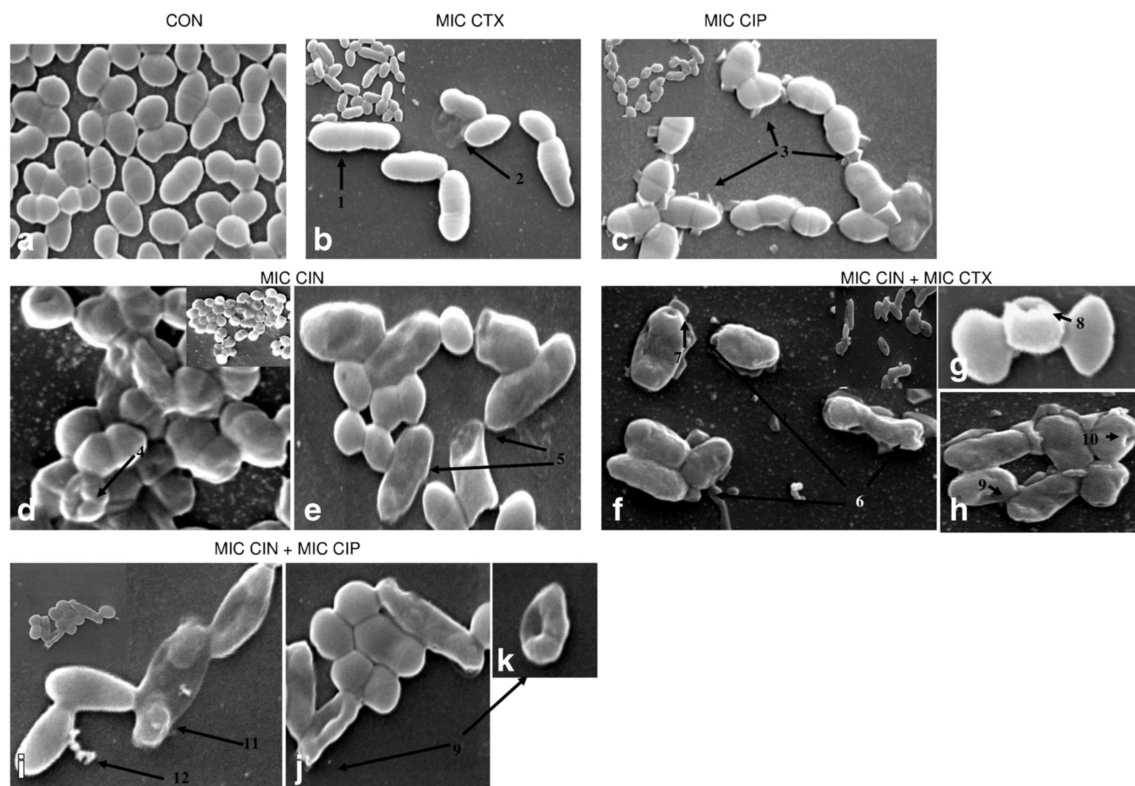

Fig. 3 Effect of cinnamaldehyde with antibiotics alone and its combination on ESBL-QR *E. coli* cellular morphology. Scanning electron micrograph of ESBL-QR *E. coli* cells treated with cinnamaldehyde (CIN), cefotaxime (CTX), and ciprofloxacin (CIP) alone and its combination. **a** Untreated *E. coli* cells showing an intact, regular oval shape with smooth cell membrane. **b**, **c** *E. coli* cells grown in the presence of MIC CTX/CIP. 1, elongated; 2, receding of cytoplasmic membrane; 3, rough cell membrane with some particulate material. **d**, **e** *E. coli* cells were grown in the presence of MIC CIN. 4, pore and crumpled on cell surface; 5, diminishing of cytoplasmic membrane and empty cells. **f**–**h** *E. coli* cells grown in the presence of combination of MIC CIN with CTX. 6, leakage and cell debris depositon on cell surfaces due to the bursting of cells were visible; 7–10, deep pore with rough irregular cell membrane. **i**, **j** *E. coli* cells grown in the presence of combination of MIC CIN with CIP. 11, receding of cytoplasmic content from cell membrane and crumbling of cellular content; 12, deposition of lytic material in a form of vesicles; 13, large groove

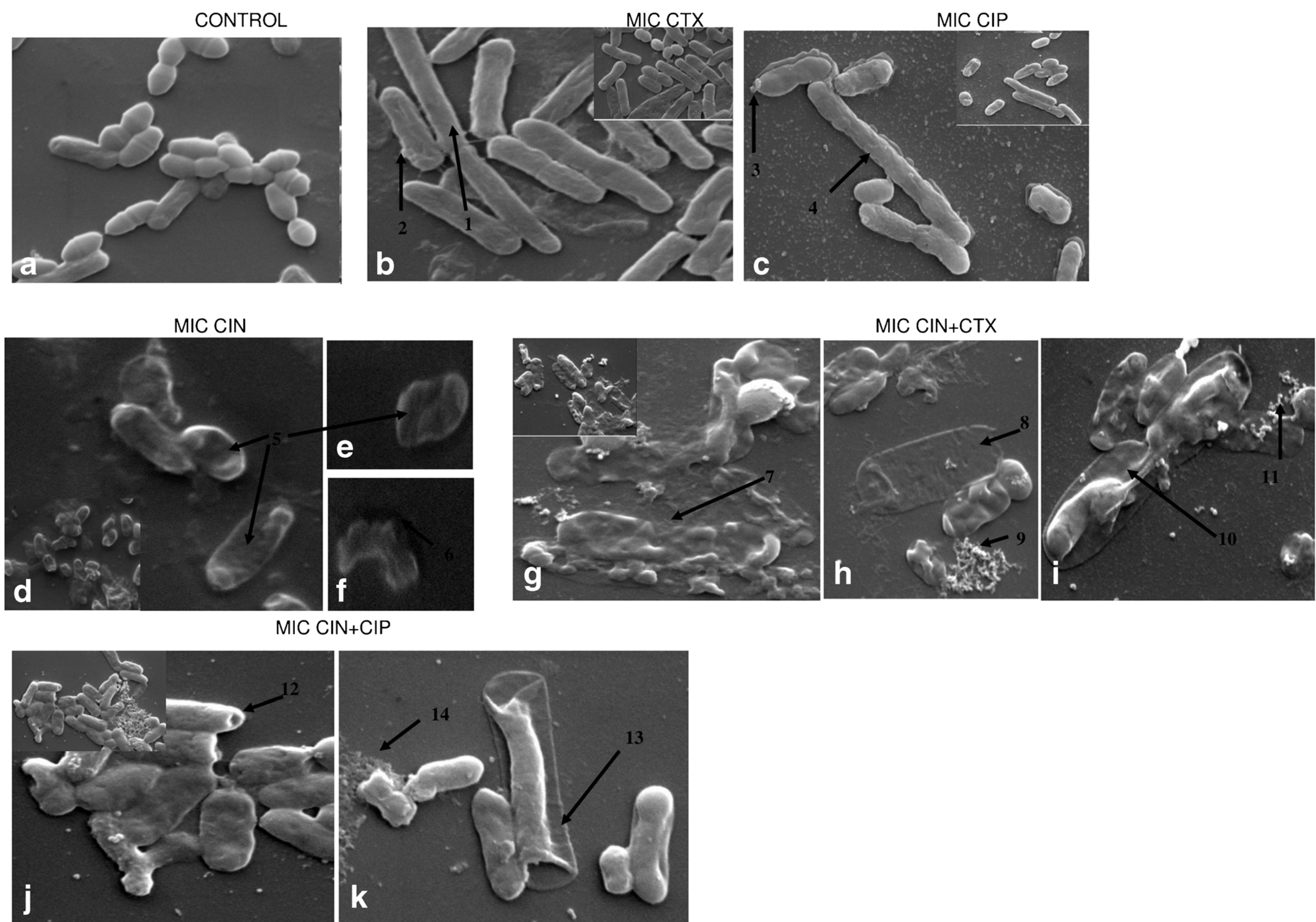

Fig. 4 Effect of cinnamaldehyde with antibiotics alone and its combination on ESBL-QR *K. pneumoniae* cellular morphology. Scanning electron micrograph of ESBL-QR *K. pneumoniae* cells treated with cinnamaldehyde (CIN), cefotaxime (CTX), and ciprofloxacin (CIP) alone and its combination. **a** Untreated *K. pneumoniae* cells showing an intact, regular oval shape with smooth cell membrane. **b**, **c** *K. pneumoniae* cells grown in the presence of MIC CTX/CIP. 1–4, elongated; 2–3, rough cell membrane with some particulate material. **d**–**f** *K. pneumoniae* cells grown in the presence of MIC CIN. 5, convoluted surfaces with loosened cell membrane; 6, shrinkage cell membrane

E. coli and *K. pneumoniae* were noticed on the treatment with CIN alone, indicating permeability changes that created osmotic inequity leading to cell lysis. Similar observations have also been reported in previous works on *E. coli*–ATCC8735 and S17 treated with cinnamaldehyde [21, 22].

Treatment with CIN and CTX/CIP revealed deep pore, disruption of cytoplasmic membrane, and decomposition of inner organelles on cell surfaces indicating autolysis leading to cell death, validating their synergistic relationship (Fig. 3f–k). These effects have been previously documented as converging effect of exogenous and endogenous oxidative stress generated by bioactive compound and cefotaxime which instigated membrane degradation with shrunken cell wall and disruption of cellular proteins [17, 23].

A significant change in expression level of outer membrane porins and efflux pump was noticed between CIN-treated and untreated control cell (Fig. 5a, b and Table 1). However, the change in beta-lactamase genes' expression level due to CIN exposure was gene-specific (Fig. 5c and Table 1). *OmpC* was upregulated in the presence of CIN/CTX alone and in combination with CTX-CIP. Overexpression of *OmpF* was noticed after CIN/CTX/CIP-alone treatment and with CTX-CIP combination, respectively.

OmpK35 was upregulated after treatment with CIN/CIP alone and using CTX/CIP and CIN combination. *OmpK36* was upregulated in the presence of CIN, CTX, CIP alone, and after treatment with combination of CIN with CIP. Thus, upregulation of *Omp* expressions results in alteration of porin channels that might have decreased antibiotic resistance profile. Similar phenomenon was demonstrated with the upregulation of *ompF* in the presence of aqueous extract of *Aegle marmelos* fruit which allowed greater permeability of beta-lactam antibiotics among *Enteropathogenic E. coli* and multidrug-resistant *Shigella dysenteriae* and *S. flexneri* [24, 25].

Transcription levels of *acrB* were downregulated in the presence of CIN/CIP alone and CTX/CIP and CIN combination (Fig. 5b). Previous studies also documented downregulation of *adeAB* and *AcrAB-TolC* efflux pumps' expressions

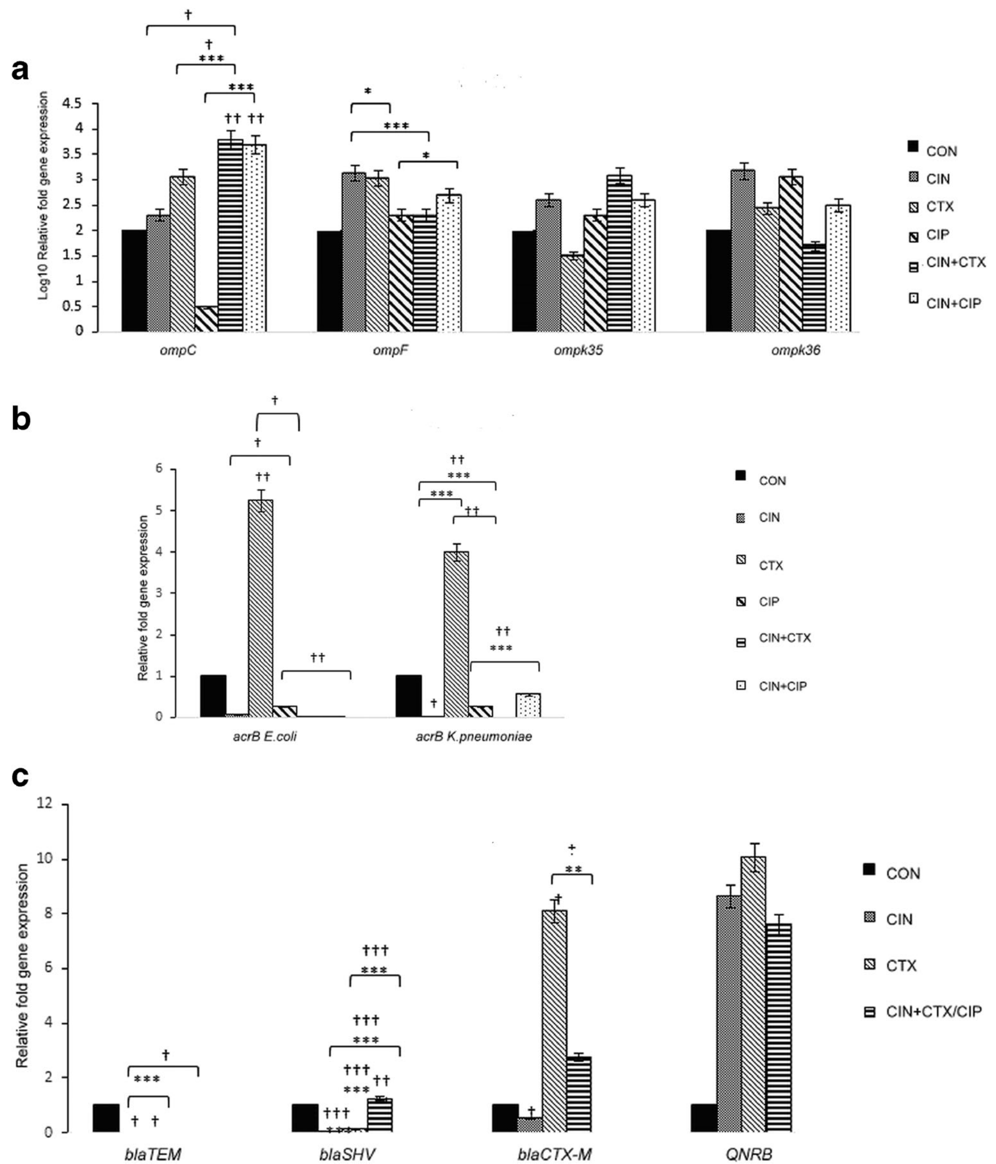

Fig. 5 Effect of cinnamaldehyde with antibiotics alone and its combination on gene expression profile. Effect of cinnamaldehyde (CIN), cefotaxime (CTX), and ciprofloxacin (CIP) alone and its combination on gene expression profile. **a** Analysis of the expression of *ompF*, *ompC*, *ompK35*, and *ompK3*. **b** Analysis of the expression of *acrB* (*E. coli*) and *acrB* (*K. pneumoniae*). **c** Analysis of the expression of *blaTEM*, *blaSHV*, *blaCTX-M*, and *QNRB* gene. Asterisks indicate level of significance of a two-tailed Student's *t* test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Daggers indicate level of significance of ANOVA ($^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, $^{\dagger\dagger\dagger}P \leq 0.001$)

Table 1 Expressional fold changes of different genes in real-time RT-PCR

	Fold changes of gene expression in relation to untreated control					
	Gene name	CIN	CTX	CIP	CIN + CTX	CIN + CIP
Porins	*ompC*	2.01 ↑[a]	10.13 ↑ [a]	0.03 ↓ [b]	59.7 ↑[a]	52.7 ↑[a]
	ompF	14 ↑[a]	11 ↑[a]	2.2 ↑[a]	2 .2 ↑[a]	55.9 ↑[a]
	ompK35	5 ↑[a]	0.4 ↓[b]	2.1 ↑[a]	13 ↑[a]	4 ↑[a]
	ompK36	16 ↑[a]	3 ↑[a]	12 ↑[b]	0.5 ↓[b]	4 ↑[a]
Efflux pump	*acrB–E. coli*	13.8 ↓[b]	0.2 ↑[a]	36.7 ↓[b]	200 ↓[b]	104 ↓[b]
	acrB–K. pneumoniae	125 ↓[b]	0.3 ↑[a]	3.8 ↓[b]	400 ↓[b]	1.6 ↓[b]
Antibiotic resistance gene	*blaTEM*	142 ↓[b]	312 ↓[b]	NA	250 ↓[b]	NA
	blaSHV	357 ↓[b]	8 ↓[b]	NA	1.27 ↓[b]	NA
	blaCTXM	1.8 ↓[b]	8.1 ↑[a]	NA	2.8 ↑[a]	NA
	QnrB	8.6 ↑[a]	NA	10.1 ↑[a]	NA	7.6 ↑[a]

CIN, cinnamaldehyde; *CTX*, cefotaxime; *CIP*, ciprofloxacin; *NA*, not applicable

[a] ↑ = upregulation

[b] ↓ = downregulation

among *Acinetobacter baumannii* and *E. coli* by cinnamaldehyde and total alkaloids [26, 27].

BlaTEM expression was noticeably inhibited after treatment with CIN and CTX respectively and with their combination. CIN also inhibited *blaSHV* and *blaCTX-M* expression (Fig. 5c). In contrast, no significant change was noticed in *QNRB* expression level after treatment with CIN and CIP. A similar reduction in the expression of these genes was demonstrated by baicalein against ESBL–*K. pneumoniae* [28]. All these findings suggested that efflux pump downregulation, porin overexpression, and beta-lactamase gene inhibition of ESBL-QR bacteria by cinnamaldehyde alone or in combination with traditional antibiotics might be attributed to overcoming bacterial drug resistance. Moreover, cinnamaldehyde was found to be nontoxic among intravenously treated mice previously [29]. Trans-cinnamaldehyde failed to exhibit detectable hepatocarcinogenic activity in mice. All these observations along with data obtained from the present study suggested efficient therapeutic potential of cinnamaldehyde. Thus, cinnamaldehyde seemed to enhance activities of traditional antibiotics also, thereby reducing their usage and toxicity, and combination therapy with cinnamaldehyde might eventually help to deter development of antibiotic resistance property of pathogenic bacteria.

Conclusion

Combination of cinnamaldehyde and cefotaxime/ciprofloxacin exhibited antibacterial as well as synergistic effects against ESBL-QR *E. coli* and *K .pneumoniae.* Thus, this work confirmed the therapeutic value of cinnamaldehyde against both ESBL-producing and quinolone-resistant pathogenic bacteria.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement and informed consent The study was approved by the ethical research committee (reference number: CREC-STM/53 dated 23/09/2011). Informed consent was obtained from the patients for participating in the study.

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