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A potential role of cinnamon bark essential oil and its major component in enhancing antibiotics effect against clinical isolates of extended-spectrum beta-lactamase producing *Escherichia coli*

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ABSTRACT

Escherichia coli resistance to third generation cephalosporins due to extended-spectrum-beta-lactamase (ESBL) production is a major drug-resistance issue. The present work was undertaken to investigate the phytochemical composition and antibacterial effects of cinnamon bark essential oil (CBO) and its major components individually and combined with antibiotics against ESBL-producing E. coli. CBO was obtained from pharmaceutical source and analyzed by Gas Chromatography/Mass Spectrometry (GC/MS). The antibacterial activities of CBO and its major components against ESBL-producing E. coli were assessed. The effect of combination of either CBO or its most active component with some antibiotics such as co-amoxiclav, ceftazidime, gentamicin, and ciprofloxacin against ESBL-producing E coli were evaluated. GC/MS analysis showed that the major components identified in the CBO were cinnamaldehyde (63.69%), cinnamyl acetate (9.93%), and 1,8-cineole (8.75%). The obtained results indicated that the CBO have highly significant antibacterial activity against all the tested isolates. Cinnamaldehyde proved to be the most active component of CBO, also; combination of this component or CBO with gentamicin resulted in the highest antibacterial activity, although lower dose of gentamicin was used in these combinations relative to that applied when used individually. These findings highlighted the potential role of CBO or cinnamaldehyde as an antibiotic resistance modifying agents. To our knowledge, this is the first report concerning the synergistic effect of CBO and cinnamaldehyde in combination with antibiotics against ESBL-producing E. coli isolated from clinical sources.

Keywords: *E. coli*, ESBL, Essential Oil, Cinnamon bark, Gentamicin

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INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) is a group of plasmid-borne enzymes that hydrolyze and confer resistance to the modern cephalosporin antibiotics, ESBLs confer resistance not only to penicillins, aztreonam, and cephalosporins but could also be resistant to other antibiotic classes including aminoglycosides, trimethoprim-sulfamethoxazole, and quinolones [1]. *E. coli* remains one of the main ESBL-producing organisms isolated worldwide [2], [3]. Detection of ESBL-producing *E. coli* in Egypt have been reported [4]. Current therapy for isolates of *E. coli* that express ESBLs is limited to broad-spectrum agents as imipenem [3]. However, therapeutic failures of this drug with *E. coli* strains that produce multiple β-lactamases have been reported [5]. The Infectious Diseases Society of America has listed ESBL-producing *E coli* among the six drug-resistant microbes to which new therapies are urgently needed [6]. The solution to this problem is therefore crucial and requires the search for new and more sustainable antibacterial agents.

Medicinal plants and their derivatives, such as essential oils (EOs), constitute a potential reservoir of several effective antimicrobial molecules [7]. EOs and their components have shown significant antibacterial activity against antibiotic resistant bacteria [8]–[12]. One kind of EOs is cinnamon bark essential oil (CBO) which is obtained from the inner bark of trees of the genus *Cinnamomum* [13]. The genus *Cinnamomum* (family *Laureaceae*) consists of 250 species of trees and shrubs; the most important cinnamon oils in world trade are those from *Cinnamomum verum* (formerly *C. zeylanicum*) [14]. *C. verum*, also known as Ceylon cinnamon or "true cinnamon", is indigenous to Sri Lanka and southern parts of India [13]. Accordingly, CBO and its components have shown potential antibacterial action against a wide variety of bacteria including *E. coli* [15], [16].

Combining antibiotics with EOs may lead to an increase in the antibacterial activities of both antibiotics and EOs, and may also reduce the toxic effects of both agents against mammalian cells. Many EOs and their components have demonstrated an *in vitro* ability to act synergistically with different antibiotics [17]–[21], and thus restore the activity of antibiotics that currently have reduced clinical applications owing to the development of resistance. To our knowledge there are no published reports on antibacterial activity of the CBO or its major components in combination with antibiotics against ESBL-producing *E. coli.* There is a general lack of studies investigated the activity of EOs in combination with antibiotics against ESBL- producing *E. coli*. Only one study that determined the synergistic activity of *Origanum vulgare* oil and antibiotics against MDR strains of ESBL-producing *E. coli* isolated from chicken is available [22]. Therefore, the objective of the present work is investigating the antibacterial effect of *C. verum* bark oil and its major components individually and in combination with antibiotics against ESBL-producing *E. coli*. The ultimate goal was to find a synergistic effect in order to decrease the effective dose of antibiotics on resistant bacteria, thus minimizing their potential toxic side effects and the treatment cost.

MATERIALS AND METHODS

Essential oil

CBO was obtained from a Pharaonia pharmaceutical company, Egypt. Quality of the oil was ascertained to be more than 95%. Oils were kept at 4°C in sealed air-tight glass vials covered with aluminum foil until further analysis.

Gas chromatography-mass spectrometry analysis of CBO

The analysis of CBO was performed using a Thermo Scientific ISQ Single Quadrupole GC-MS (US) equipped with a TG-5MS fused silica capillary column (1µm 0.25mm, 30m). An electron ionisation system was used with ionisation energy of 70 eV. Helium was the carrier gas at a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 280°C. Column temperature was initially at 40°C held for 3 min, then gradually increased to 280°C at a 5°C/min rate. Diluted samples (1:100 v/v, in hexane) of 1.0 µL were injected manually and splitless. The percentage (relative) of the identified compounds was computed from their GC peak areas. The components were identified based on the comparison of their relative retention time and mass spectra with those of Wiley and NIST libraries data of the GC–MS system, literature data and standards of the main components.

Antibiotics and bioactive components of CBO

Antibiotics to be tested were selected referring to CLSI document M100-S21 [23], and they are representing the antibiotics commonly used for treatment of *E. coli* infections. Antibiotic powders were obtained from pharmaceutical source and they included co-amoxiclav (Sedico co., Egypt), ceftazidime (Glaxo co., Egypt), gentamicin (Memphis/Schering co., Egypt), and ciprofloxacin (Hikma pharma co., Egypt). All powders were supplied with a stated potency (mg per g powder). They were stored in sealed containers in the dark at 4°C.

Three bioactive compounds of CBO; cinnamaldehyde, cinnamyl acetate, and 1,8-cineole were purchased from Sigma Aldrich, USA. Quality of the bioactive compounds was ascertained to be ≥ 95 pure. They were stored in sealed containers in the dark at 4°C.

Bacterial isolates

A total of 12 isolates of well–characterized ESBL-producing *E. coli* that included producers of ^{bla}CTX-M group I (4 isolates), ^{bla}CTX-M group I plus ^{bla}TEM (7 isolates), and ^{bla}CTX-M group IV plus ^{bla}TEM (1 isolates) were used in this study. These isolates were previously characterized [4] and were found to be resistant to the following antibiotics; cephalothin, cefuroxime, co-amoxiclav, cefotaxime, ceftazidime, ceftriaxone, cefepime, gentamicin, ciprofloxacin, and co-trimoxazole. All isolates were stored in glycerol at −20°C and recovered in Tryptic Soy agar (TSA) by incubation for 24 h at 37°C.

Antibacterial assay of cinnamon bark oil and its bioactive components on ESBL-producing *E. coli*

The antibacterial property of CBO and its major components was determined by agar-well diffusion method [24]. The bacterial cultures were grown in Muller Hinton broth medium at 37°C until they reached about 10^8 CFU/mL.

Cultures were then diluted (10-fold) in physiological saline solution (0.9% w/w) and 100 µl of each bacterial culture were inoculated onto the surface of Muller Hinton agar plate. Wells of 5 mm diameter were made in the solidified agar using a Pharmacia gel punch and filled with 50 µL of the undiluted CBO, aqueous dimethyl sulfoxide (DMSO) solutions of; cinnamaldehyde, cinnamyl acetate, and 1,8-cineole individually and in combinations (concentrations used for bioactive compounds are the same their relative contents in the CBO as indicated by GC-MS results), and DMSO solvent blank. All Petri dishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test oils/ compounds. The plates were left for 30 min at 4°C to allow the diffusion of oil and bioactive compounds, and then they were incubated at 37°C for 18 h. After the incubation period, zone of inhibition was measured in mm.

MIC determination

The minimum inhibitory concentration (MIC) values of the bacterial isolates under study against antibiotics were determined by the agar dilution method [25]. The concentrations of antibiotics in agar dilution plates ranged from 1 to 512 (µg/mL). Three to five well-isolated *E. coli* colonies were selected from a 24 h TSA plate culture, and transferred into a tube containing 5 mL of Tryptic Soy broth, which incubated at 37°C until it achieved the turbidity of the 0.5 McFarland standard. This bacterial suspension of *E. coli* containing $1x10^7$ CFU/mL was used to inoculate the plates. Inoculated plates were incubated at 35°C for 24 h. MIC was defined as the lowest concentration that did not result in any visible growth of the microorganism compared with the growth in the control plate. The MIC results were interpreted as referred by CLSI document M100-S21 [23]; the isolates were reported as susceptible, intermediate, or resistant to the antibiotics that have been tested. *E. coli* ATCC 25922 was used as a control strain to validate susceptibility tests was.

The MIC values of CBO and the most active component against tested bacterial isolates were determined by the agar dilution method described for essential oils [26], a final concentration of 0.5 % tween 20 (Sigma) (v/v) was incorporated into the agar medium before autoclaving to enhance oil solubility. The concentration of CBO and the most active component in the medium ranged from 0.15 to 10 (mg/mL). Plates were dried at 35°C for 30 min prior to inoculation with 1–2 µL spots containing 1×10^4 CFU of each isolate, using a multipoint replicator. Muller Hinton agar plate, with 0.5% (v/v) tween-20 and without oil, was used as a positive growth control. Inoculated plates were incubated at 35°C for 24 h. All determinations were performed in triplicates.

Synergistic test

The checkerboard assay was carried out according to a published report [27]. Serial, two-fold dilutions of the CBO or the most active component in combination with antibiotics (i.e. co-amoxiclav, ceftazidime, gentamicin, and ciprofloxacin) were prepared to assess the antibacterial activity of these combinations against bacterial isolates under test. The concentrations prepared were $^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$ of the corresponding MIC values for each agent. All determinations were performed in triplicates. The analysis of the combination was obtained by calculating the FIC index (FIC_I) as follows:

$$FIC_I = \frac{MIC_{A/B}}{MIC_A} + \frac{MIC_{B/A}}{MIC_B}$$

Where – (A) is the CBO or the most active component and – (B) is the antibiotic under test. The FIC_I was interpreted as: (I) a synergistic effect when $FIC_I \leq 0.5$; (II) partial synergy effect when $FIC_I > 0.5$ and < 1.0; (III) an additive effect when $FIC_I = 1$; (IV) an indifferent effect when $FIC_I > 1.0$ and < 4.0; and (VI) an antagonistic effect when $FIC_I > 4$.

Based on FIC index results, we further studied the killing curve to confirm synergistic activity of the combinations [27]. The MIC of each compound that gave synergistic FIC index of combination was chosen for this investigation. Antibiotics and CBO were tested individually and in combination at sub-MIC level (The sub-inhibitory concentration between FIC and MIC value). Each flask contained a final volume of 20 mL cation adjusted Muller Hinton broth supplemented with 0.5 % tween 80 to enhance the oil solubility [28]. The mixtures were inoculated with a broth culture of the isolate under test adjusted to give approximately 5×10^5 CFU/mL. After 0, 3, 6, and 24 h of incubation at 37°C, aliquots were withdrawn and diluted with physiological saline solution. The dilutions were spread onto TSA and the colonies were counted after incubation at 37°C for 24 h. The number of colonies was expressed as colony forming units per milliliter (CFU/mL). The experiment was carried out in triplicates. Reduction of viable cell count ≥ 2 $\log_{10}$ after 24 h incubation in comparison with the cell count of the most active single substance was interpreted as synergy [29]

RESULTS AND DISCUSSION

Antibiotic resistance levels in *E. coli* are rapidly rising, especially with regard to quinolones and third- and fourth-generation cephalosporins [30]. ESBLs enzymes are the most common mechanism of antibiotic resistance in *E. coli* [31]. The problem of increasing resistance of ESBL producing *E. coli* to different antibiotics; necessitated the search for safe and effective antibacterial agents that may be used to treat persistent bacterial infections, a feasible approach is to use essential oils as alternative agents. Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens [32]. This study has evaluated the chemical composition and antibacterial activities of *C. zeylanicum* bark oil and its major components individually and in combination with antibiotics against clinical isolates of ESBL producing *E. coli*.

Gas chromatography-mass spectrometry analysis of CBO

In general, the compounds present in the CBO are cinnamaldehyde, camphor, cinnamyl-acetate, caryophyllene, trans α-bergamotene, caryophyllene oxide, linalool, geraniol, bornyl acetate, α-cubebene, γ-elemene, α-copaene, guaiol, and eugenol [14]. In this study; 17 compounds were identified in the CBO, representing 97.99 % of the total oil. Table (1) shows the components of CBO listed in order of their elution on the TG-5MS column. The major compounds in the essential oil were cinnamaldehyde (63.69%), cinnamyl acetate (9.93%), and 1,8-cineole (8.75%). Minor components identified were α-terpineol (3.47%), α-pinene (3.12%), sabinene (2.44 %), and terpinen-4-ol (2.22%). Other components analyzed in the oil were present in amounts less than 1 %. Unlu and co-workers have reported a slightly different composition for CBO. The major compounds in the essential oil were cinnamaldehyde (68.95%), benzaldehyde (9.94%), and cinnamyl acetate (7.44%). Other components analyzed in the oil were α-pinene, 1,8-cineole, linalool, eugenol and cinnamic acid [16]. As previously reported, many factors such as the geographical origin, genetic factors, plant material and season at which the plants were collected may be responsible for the chemical composition of the EOs [14].

Table (1): Chemical composition of CBO

Peak	Retention time	Compounds	% of relative content
1	10.64	α-Pinene	3.12
2	11.15	Camphene	0.62
3	12.25	Sabinene	2.44
4	14.45	1,8-Cineole	8.75
5	19.56	endo-Borneol	0.69
6	20.02	Terpinen-4-ol	2.22
7	20.61	α-Terpineol	3.47
8	24.21	Cinnamaldehyde	63.69
9	26.80	α-Copaene	0.46
10	28.03	trans-α-Bergamotene	0.39
11	28.22	trans-Caryophyllene	0.58
12	29.23	Cinnamyl acetate	9.93
13	30.71	α-Muurolene	0.34
14	31.40	ë-Cadinene	0.45
15	33.22	Caryophyllene oxide	0.20
16	34.93	tau.-Muurolol	0.46
17	35.29	α-Cadinol	0.22
Total identified compounds			97.99

Antibacterial assay of cinnamon bark oil and its bioactive components on ESBL-producing *E. coli*

Antibacterial activity of CBO and its three major components in terms of inhibition zone are presented in Table (2). The results revealed that CBO had a highly active antibacterial behavior against all tested isolates with inhibition zone diameters varying from 27 to 30 mm (Table 2). Several studies have reported that CBO exhibited a significant antibacterial activity against *E. coli* [16], [33], [34]. A high level of antibacterial activity of CBO against ESBL-producing *E. coli* was also reported [35], [36]. It was proposed that the essential oils affect microbial cells by various mechanisms, including attacking the phospholipid bilayer of the cell membrane, disrupting enzyme systems, compromising the genetic material of bacteria, and forming fatty acid hydroperoxidase caused by oxygenation of unsaturated fatty acids [37], [38].

To investigate the role of the major components of the CBO in antibacterial activity, the three major compounds were tested for their antibacterial activity (individually and in combination) at concentrations similar to their obtained relative contents in the GC-MS analysis of CBO. The results showed that the highest level of antibacterial activity (indicated by inhibition zones) was recorded for cinnamaldehyde when tested individually or in combination with other components (Table 2). These findings are quite similar to that of other reports [34], [35]. It has been proposed that cinnamaldehyde cause membrane disruption of bacterial cell by inhibition of ATPase activity [39]. Comparing the antibacterial activity of cinnamaldehyde with the activity of CBO, indicated that the inhibitory effect

of cinnamaldehyde individually and in combination with cinnamyl acetate and 1,8 cineole is less than that of CBO against all test isolates. So we concluded that the inhibitory properties of CBO against these isolates are not solely due to cinnamaldehyde. It could be a synergistic effect derived from some components in CBO endows it with such potent antibacterial activity. Some authors suggested that the components present in the greatest proportions are not necessarily responsible for the total antimicrobial activity; the involvement of less abundant components should also be considered [40]. Since cinnamaldehyde exhibited the best antibacterial activity among the major component of CBO, it was further tested individually and in combination with antibiotics against ESBL producing *E. coli*.

Table (2): Inhibition zone diameters of CBO and its major components against ESBL-producing *E. coli* isolates

Isolates	Inhibition zone diameters (mm)*							
	[1]CBO	[2]CIN	[3]Cinn. acet.	[4]Cle.	2+3	2+4	3+4	2+3+4
1	29	22	0	0	24	23	0	26
9	29	21	0	0	23	22	0	25
15	27	20	0	0	23	22	0	23
25	28	21	0	0	24	22	0	24
26	28	22	0	0	24	23	0	25
30	30	20	0	0	23	23	0	26
57	27	21	0	0	22	22	0	24
59	30	23	0	0	25	24	6	26
63	30	22	0	0	24	22	6	25
78	28	20	0	0	23	21	0	24
81	29	21	0	0	23	22	0	25
89	29	22	0	0	24	22	0	24

**50 µL of undiluted cinnamon bark oil and its major bioactive compounds (individually and in combination) were applied to agar plates containing ESBL producing E. coli.*

[1] CBO (concentration 100%); [2] Cinnamaldehyde (concentration 63%); [3] Cinnamyl acetate (concentration 9%); [4] 1,8-cineole (concentration 8%)

MIC determination

The MIC values of the tested antibiotics, CBO and cinnamaldehyde are shown in Table (3). The MIC values of the tested antibiotics indicated that the ESBL-producing *E. coli* isolates have an alarming resistance levels to multiple drugs (Table 3). MICs values obtained in this study for ESBL producing *E. coli* isolates against the tested antibiotics were very high compared to the MICs values obtained for the same antibiotics in other studies [41]–[44]. Our data indicate that the situation is more serious than those reported in other countries; this would result in treatment difficulties for diseases caused by these bacteria. The MIC values of CBO and cinnamaldehyde are the same; both of them have MIC values of 0.6 mg/mL against all tested bacteria. Similarly, Ooi and co-workers reported that the antimicrobial effectiveness of Chinese cinnamon oil and its major components (cinnamaldehyde) against various isolates of bacteria are almost equivalent [45].

Table (3): Minimum inhibitory concentration of antibiotics, CBO, and cinnamaldehyde against ESBL-producing *E. coli* isolates

Isolates	MIC					
	Antibiotics[1] (µg/mL)				CBO (mg/mL)	CIN (mg/mL)
	AUG	CAZ	GN	CIP		
	≥32/16*	≥16*	≥16*	≥4*		
1	32/16	16	64	16	0.6	0.6
9	128/64	128	32	128	0.6	0.6
15	32/16	64	64	64	0.6	0.6
25	32/16	16	32	16	0.6	0.6
26	128/64	128	64	256	0.6	0.6
30	32/16	32	32	512	0.6	0.6
57	32/16	16	64	32	0.6	0.6
59	32/16	16	32	16	0.6	0.6
63	32/16	16	64	32	0.6	0.6
78	64/32	64	64	512	0.6	0.6
81	256/128	128	32	256	0.6	0.6
89	32/16	64	32	16	0.6	0.6

AUG, Co-amoxiclav; CAZ, Ceftazidime; GN, Gentamicin; CIP, Ciprofloxacin; CIN, Cinnamaldehyde

**Resistance breakpoints according to CLSI document M100-S21 (CLSI, 2011)*

Synergistic test

The combination of EOs or their components with antibiotics is one of the novel ways to overcome the resistance mechanisms of bacteria. In the present study, the synergistic effects of CBO or cinnamaldehyde with different antibiotics were demonstrated against ESBL-producing *E. coli* and the results are presented in Tables (4, and 5), respectively. To the best of our knowledge, this is the first report concerning the synergistic effects of CBO or cinnamaldehyde in combination with antibiotics against ESBL-producing *E. coli* isolated from clinical sources. In combination of CBO with co-amoxiclav, FIC indices obtained for this combination indicated the occurrence of partial synergistic effect against 6/12 of the tested isolates, and additive effect against 6 of the tested isolates. While in combination of CBO with ceftazidime, FIC indices obtained for this combination indicated the occurrence of partial synergetic effect against 8/12 of the tested isolates, additive effect against 2 of the tested isolates, and indifferent effect against 2 of the tested isolates. The best antibacterial activity was obtained with the combination of CBO and gentamicin, the results obtained highlight the occurrence of pronounced synergism in which CBO enhanced the action of gentamicin at lower dose (8 µg/mL) compared to gentamicin when tested individually (32 µg/mL) against two *E. coli* isolates no. 30, and 59. In addition, partial synergetic and additive effects were obtained with this combination against 8, and 2 of the tested isolates, respectively. Combination of CBO with ciprofloxacin indicated mainly "indifferent effect" (Table 4).

Table (4): Combination of CBO and antibiotics against ESBL-producing *E. coli* isolates

Isolates	FIC index of different combinations of CBO with antibiotics			
	CBO + AUG	CBO + CAZ	CBO + GN	CBO + CIP
1	1.0[c]	0.75[b]	0.75[b]	1.25[d]
9	0.75[b]	0.75[b]	0.75[b]	1.5[d]
15	0.75[b]	1.0[c]	0.62[b]	1.5[d]
25	0.75[b]	1.25[d]	1.0[c]	1.25[d]
26	0.75[b]	0.75[b]	0.75[b]	1.5[d]
30	1.0[c]	0.75[b]	0.5[a]	1.5[d]
57	1.0[c]	1.25[d]	0.75[b]	1.5[d]
59	0.75[b]	0.75[b]	0.5 [a]	1.5[d]
63	1.0[c]	1.0[c]	0.75[b]	2.0[d]
78	0.75[b]	0.75[b]	1.0[c]	2.0[d]
81	1.0[c]	0.75[b]	0.75[b]	1.5[d]
89	1.0[c]	0.62[b]	0.75[b]	1.25[d]

CBO, Cinnamon bark oil; AUG, Co-amoxiclav; CAZ, Ceftazidime; GN, Gentamicin; CIP, Ciprofloxacin
[a] Synergism; [b] Partial synergism; [C] Additive; [d] Indifferent

Results for the antibacterial activity of a combination of cinnamaldehyde with antibiotics are very similar to the results for a combination of CBO with antibiotics. In combination of cinnamaldehyde with co-amoxiclav, FIC indices obtained for this combination indicated the occurrence of partial synergistic effect against 5/12 of the tested isolates, and additive effect against 7 of the tested isolates. While in combination of cinnamaldehyde with ceftazidime, FIC indices obtained for this combination indicated the occurrence of partial synergetic effect against 3/12 of the tested isolates, additive effect against 7 of the tested isolates, and indifferent effect against 2 of the tested isolates. Similar to CBO, the best antibacterial activity is obtained with the combination of cinnamaldehyde and gentamicin, the results obtained highlight the occurrence of pronounced synergism in which cinnamaldehyde enhanced the action of gentamicin at lower dose (8 µg/mL) compared to gentamicin when tested individually (32 µg/mL) against two *E. coli* isolates no. 30, and 89. In addition, partial synergetic and additive effects were obtained with this combination against 8, and 2 of the tested isolates, respectively. Combination of cinnamaldehyde with ciprofloxacin indicated mainly "indifferent effect" (Table 5).

Table (5): Combination of cinnamaldehyde and antibiotics against ESBL-producing *E. coli*

Isolates	FIC index of different combinations of CIN with antibiotics			
	CIN + AUG	CIN + CAZ	CIN + GN	CIN + CIP
1	1.0[c]	1.0[c]	1.0[c]	1.5[d]
9	1.0[c]	1.0[c]	0.75[b]	1.5[d]
15	0.75[b]	1.0[c]	0.62[b]	1.25[d]
25	1.0[c]	1.25[d]	0.75[b]	1.5[d]
26	0.75[b]	1.0[c]	1.0[c]	1.5[d]
30	1.0[c]	0.75[b]	0.5[a]	1.5[d]
57	1.0[c]	1.25[d]	0.75[b]	1.5[d]
59	0.75[b]	0.75[b]	0.75[b]	1.5[d]
63	1.0[c]	1.0[c]	0.62[b]	1.5[d]
78	0.75[b]	1.0[c]	0.62[b]	1.5[d]
81	1.0[c]	1.0[c]	0.75[b]	1.5[d]
89	0.75[b]	0.75[b]	0.5[a]	1.25[d]

CIN, Cinnamaldehyde; AUG, Co-amoxiclav; CAZ, Ceftazidime; GN, Gentamicin; CIP, Ciprofloxacin
[a] Synergism; [b] Partial synergism; [C] Additive; [d] Indifferent

The synergistic effect of CBO with gentamicin against ESBl-producing *E. coli* isolates 30 and 59 was confirmed by time-kill curve experiments. The cultures of both isolates, with a cell density of 5×10^5 CFU/mL, were exposed to sub-MIC of gentamicin (8 µg/mL), or CBO (0.15 µL/mL) individually, and combination of CBO with gentamicin at the same concentrations (Figures 1, and 2). The results showed that the viable counts of both isolates were slightly reduced in the presence of CBO compared with the untreated control culture between 6 and 24 h incubation. Gentamicin decreased the cell viable counts of both tested isolates to 1×10^4 CFU/mL, 1×10^3 CFU/mL after 6, 24 h incubation, respectively. Combination of CBO and gentamicin resulted in a synergistic effect with >2 $\log_{10}$ decrease of colony count for both tested isolates after 6 h and 24 h compared to the most active single substance (gentamicin). These results confirmed the synergistic combination of CBO and gentamicin against the tested isolates.

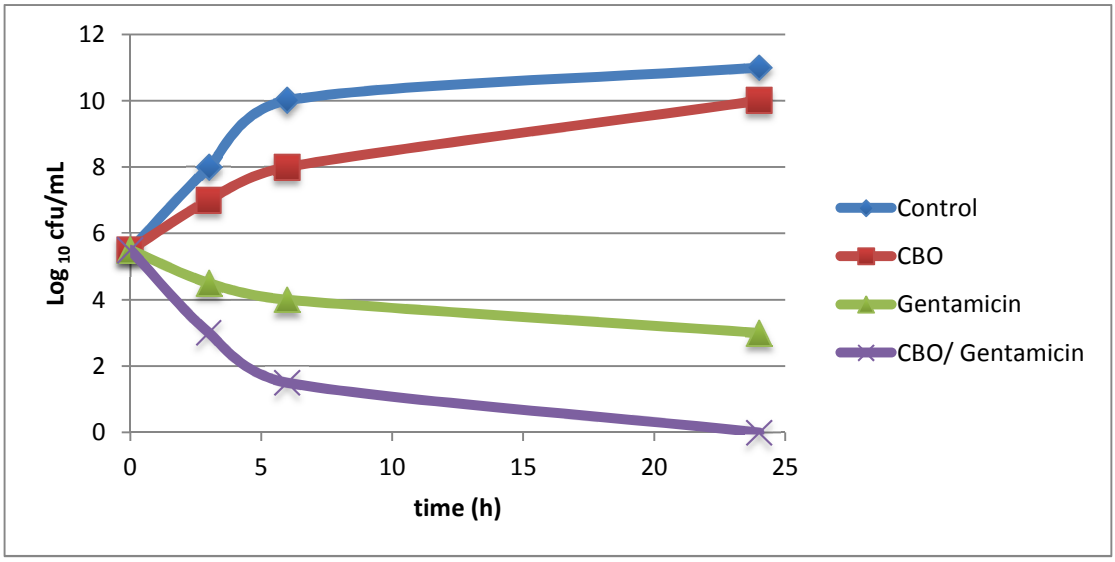

Figure (1): Killing curve of gentamicin (8 µg/mL), CBO (0.15 µL/mL), and gentamicin (8 µg/mL) and CBO (0.15 µL/mL), against ESBL-producing *E. coli* isolate no. (30)

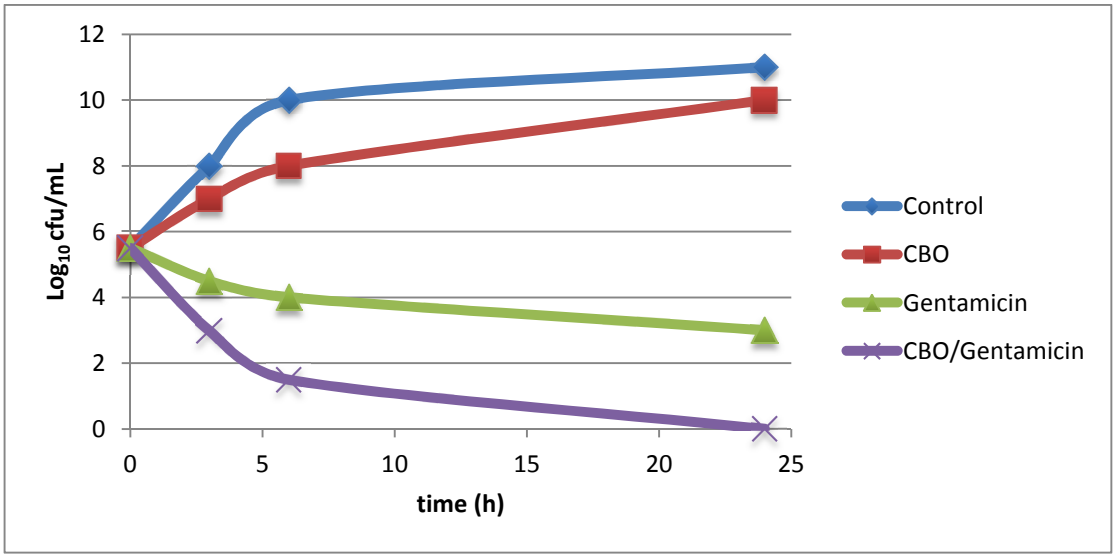

Figure (2): Killing curve of gentamicin (8 µg/mL), CBO (0.15 µL/mL), gentamicin (8 µg/mL) and CBO (0.15 µL/mL), against ESBL-producing *E. coli* isolate no. (59)

The synergistic effect of cinnamaldehyde with gentamicin against ESBL-producing *E. coli* isolates 30, and 89 was also confirmed by killing-curve experiments. The cultures of both isolates, with a cell density of 5×10^5 CFU/mL, were exposed to sub MIC of gentamicin (8 µg/mL), or cinnamaldehyde (0.15 mg/mL) individually, and combination of gentamicin with cinnamaldehyde at the same concentrations (Figures 3, and 4). The results showed that the viable counts of both isolates were slightly reduced in the presence of cinnamaldehyde compared with the untreated control culture between 6 and 24 h incubation. Gentamicin decreased the cell viable counts of both tested isolates to 1×10^4 CFU/mL, 1×10^3 CFU/mL after 6, and 24 h incubation, respectively. Combination of cinnamaldehyde and gentamicin resulted in a synergistic effect with >2 $\log_{10}$ decrease of colony count of both the tested isolates after 6 h

and 24 h compared to the most active single substance (gentamicin). These results confirmed the synergistic antibacterial activity of cinnamaldehyde/ gentamicin combination against the tested isolates.

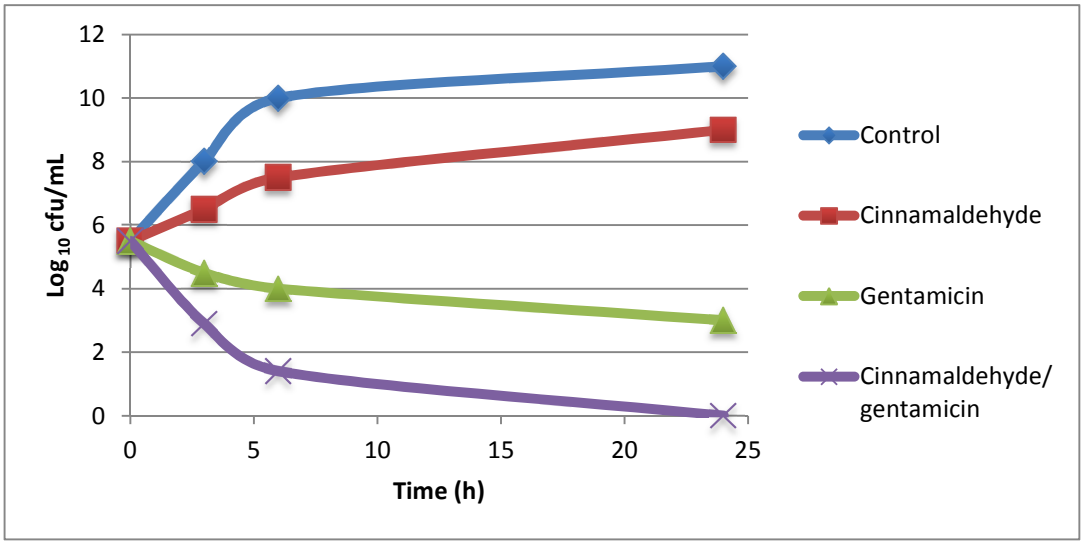

Figure (3): Killing curve of cinnamaldehyde (0.15 mg/mL), gentamicin (8 µg/mL), and cinnamaldehyde (0.15 mg/mL) and gentamicin (8 µg/mL) against ESBL-producing isolate no. (30)

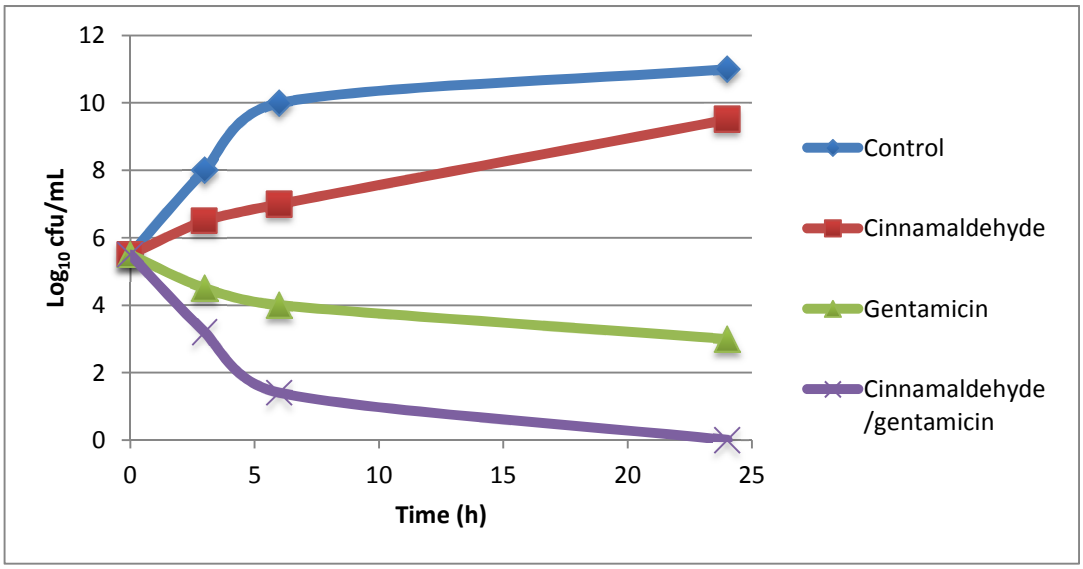

Figure (4): Killing curve of cinnamaldehyde (0.15 mg/mL), gentamicin (8 µg/mL), and cinnamaldehyde (0.15 mg/mL) and gentamicin (8 µg/mL) against ESBL-producing isolate no. (89)

Several studies have reported a synergistic interaction of EOs or their components with antibiotics against *E. coli* [18], [46]–[48]. One study has evaluated the combined effect of CBO and β-lactam antibiotics against β-lactamase producing *E. coli* [49]. The authors of that study reported a synergistic effect only for CBO/piperacillin combination, but not for any of the other tested combinations. In another study, researchers reported that cinnamaldehyde was highly effective in reducing the resistance of *E. coli* to ampicillin, tetracycline, penicillin, erythromycin and novobiocin [19]. Similar to our findings, many studies have reported synergistic activity for gentamicin and EOs or their components against different bacterial species including *E. coli* [20], [29], [50], [51]. It was proposed that the main mode of action of CBO could be attributed to the disruption of the bacterial membrane both at lethal and sub-lethal concentrations, subsequently increasing the nonspecific mobility of the antibiotic into the bacterial cell [52].

CONCLUSION

The present study reports the potential role of CBO and its major component (cinnamaldehyde) in enhancing the activity of some antibiotics against ESBL-producing *E. coli* isolates. Based on the obtained results, combination of CBO or cinnamaldehyde with gentamicin had synergistic effect against ESBL-producing *E. coli*. Application of

these combinations could be promising in reduction of the minimum effective dose of the drugs, thus minimizing their possible toxic side effects and the treatment cost. Thus, use of CBO or cinnamaldehyde individually and in combination with other antibacterial agents, may provide a promising new scheme in phytotherapy.

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