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Antibacterial Mode of Action of *Cinnamomum verum* Bark Essential Oil, Alone and in Combination with Piperacillin, Against a Multi-Drug-Resistant *Escherichia coli* Strain

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This study aims to investigate the mechanism of action of the cinnamon bark essential oil (CB), when used singly and also in combination with piperacillin, for its antimicrobial and synergistic activity against beta-lactamase TEM-1 plasmid-conferred *Escherichia coli* J53 R1. Viable count of bacteria for this combination of essential oil and antibiotic showed a complete killing profile at 20 h and further confirmed its synergistic effect by reducing the bacteria cell numbers. Analysis on the stability of treated cultures for cell membrane permeability by CB when tested against sodium dodecyl sulfate revealed that the bacterial cell membrane was disrupted by the essential oil. Scanning electron microscopy observation and bacterial surface charge measurement also revealed that CB causes irreversible membrane damage and reduces the bacterial surface charge. In addition, bioluminescence expression of *Escherichia coli* [pSB1075] and *E. coli* [pSB401] by CB showed reduction, indicating the possibility of the presence of quorum sensing (QS) inhibitors. Gas-chromatography and mass spectrometry of the essential oil of *Cinnamomum verum* showed that *trans*-cinnamaldehyde (72.81%), benzyl alcohol (12.5%), and eugenol (6.57%) were the major components in the essential oil. From this study, CB has the potential to reverse *E. coli* J53 R1 resistance to piperacillin through two pathways; modification in the permeability of the outer membrane or bacterial QS inhibition.

Keywords: *Cinnamomum verum*, essential oil, chemical composition, antibacterial activity, membrane permeability, quorum sensing

Introduction

In our previous study, we combined the beta-lactam antibiotics with essential oils to examine their *in vitro* antimicrobial activity against plasmid-conferred multi-drug-resistant bacteria. The result highlighted a pronounced synergism between piperacillin and cinnamon bark (*Cinnamomum verum* (cort.)) essential oil (CB) against beta-lactamase TEM-1 bacteria, resulting in a fractional inhibitory index (FIC) of 0.5 [37].

Essential oils consist of about 20 to 60 components that are highly complex and volatile, and are secondary metabolites produced by aromatic plants. A study on the combination effects of commercially available cinnamon oil and antibiotics (doxycycline hyclate, metronidazole, and ciprofloxacin hydrochloride) was carried out against *Staphylococcus aureus*, but the mode of action is unknown [21]. The antibacterial activity of cinnamon combined with clove essential oils in vapor phase wielded a synergistic effect for the inhibition of *Listeria monocytogenes*, *Bacillus cereus*, and *Yersinia enterocolitica* [14]. Studies on the use of cinnamon oil in the food industry in preventing food spoilage and foodborne diseases were also reported [3, 27]. There are many factors contributing to the antimicrobial activity of essential oils,

such the composition and structure as well as their functional groups. Eugenol, one of the commonly found major components in cinnamon essential oil, has been shown to exhibit synergistic interaction against gram-negative bacteria when tested with antibiotics, where alteration of membrane permeability was the primary mode of action [9, 15]. Despite numerous reports on the efficacy of CB as an antibacterial agent, its mechanism of action has been documented relatively poorly. The vast range of different groups of chemical compounds present in one essential oil suggests that antibacterial activities may be contributed by more than one specific mechanism or component. Hence, there may be several targets in a cell playing roles in potentiating influence. Since understanding its mode of action is essential in combating the onslaught of microbial resistance, this study was carried out to investigate the mode of action of CB alone and in combination with piperacillin against a model bacterium.

Materials and Methods

Cinnamon Bark Essential Oil and Antibiotic

The cinnamon bark essential oil (*Cinnamomum verum* (cort.)) was purchased from Aroma Trading Ltd. (Milton Keynes, UK). Piperacillin (Sigma Aldrich, St. Louis, MO, USA) was dissolved according to the Clinical and Laboratory Standards Institute (CLSI) M100-S21 guidelines.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

An Agilent GC-MS, 7890A GC System with a triple-axis detector (5975C MSD) was used to perform the GC-MS analysis using an HP-5MS column (30 m × 250 μm × 0.25 μm). The carrier gas used was helium. An autoinjector (Agilent Technologies 7693 Autosampler) was used for sample injection at 250°C. Temperatures used for the oven column were 60°C (5 min) to 220°C at a rate of 4°C/min for 10 min, and 240°C at a rate of 1°C/min for 5 min. The column flow was 1 ml/min with a split ratio of 40:1. MS data were acquired in EI mode with scan range 30–450 *m/z*. The temperature of the MS source and MS quad was set at 250°C and 150°C, respectively. The NIST library database was used to identify the compounds. The percentage (relative) of the identified compounds was computed from their GC peak area. Only those components present in the oils in amounts higher than 0.1% were considered to be significantly present.

Bacterial Strain and Growth Conditions

The bacterial strain used in this study was *Escherichia coli* J53 R1[1], a kind gift from George A. Jacoby (Lahey Clinic, MA, USA). Mueller-Hinton broth (MHB; Oxoid, Cambridge, UK) was used for *E. coli* culture according to the CLSI M07-A8 standards.

Time-Kill Assay

A standard inoculum of approximately 10^5 CFU/ml was incubated at 37°C with agitation at 200 rpm. The test concentrations (MHB supplemented with 0.5% Tween 80) to enhance the oil solubility had been determined in our previous work [37]. The final concentrations of antibiotics and oils used were as follows: control (without treatment; with 0.5% Tween 80); CB (0.02% v/v); piperacillin (256 μg/ml); CB (0.02% (v/v)) in combination with piperacillin (256 μg/ml). Each flask contained a final volume of 10 ml. Immediately after incubation, viable counting was performed for 100 μl of samples every four-hourly until 24 h. Ten-fold serial dilutions were performed with 0.9% (w/v) sodium chloride and spread onto Mueller-Hinton agar (MHA). The plates were incubated at 37°C for 24 h. The experiment was performed in triplicates.

Outer Membrane (OM) Permeability Test

To examine the function of essential oil and antibiotic in permeating OM barriers when used singly or in combination, the methods of Hemaiswarya and Doble [15] and Marri *et al.* [22] were adapted. Overnight inoculum was harvested and suspended in PBS to OD = 0.3 at 625 nm, in preparation for the assay. For each treatment, the culture was divided into two 10 ml volumes each. Sodium dodecyl sulfate (SDS) was added to either one of the two portions. The anionic detergent SDS is excluded extracellularly by the OM under normal condition [31]. SDS functions as a permeabilizing probe, whereby it initiates cell lysis after reaching a critical extent of membrane disordering [23, 31]. Sudden influx of SDS resulting in cell death was determined at intervals of 0, 5, 10, 30, and 60 min using a UV-Vis spectrophotometer (Shimadzu Corp., Japan) [10] by measuring the decrease of optical density. The SDS used was at the final concentration of 0.1% (w/v). The experiment was carried out in triplicates.

Bacterial Surface Charge – Zeta Potential Measurement

Zeta potential was used to express cell surface charge. A Nano Zetasizer (Malvern Instruments, UK) was used to determine the zeta potential of bacterial suspensions after exposure to CB alone, piperacillin alone, and in combination. Untreated cells suspended in phosphate-buffered saline (pH 7.4) were used as the control. Electrophoretic mobility of the cells was used as a means of zeta potential. The experiment was repeated in triplicates.

Scanning Electron Microscopy

Cells after 16 h incubation in MHB at 37°C were subjected to scanning electron microscope (SEM) observation. Cells treated with CB and piperacillin singly and in combination were harvested after 5 min of exposure. The harvested cell pellet was washed with PBS (pH 7.4). Sample fixation was carried out with 4% glutaraldehyde and followed by 1% osmium tetroxide. Sequential exposure to different concentrations of acetone (35% to 100%) caused further dehydration of the samples. The samples were subsequently subjected to critical point drying, sputter-coated

Table 1. List of bacterial strains and plasmids used.

Biosensors	Description	Source
Escherichia coli [pSB401]	*luxR luxl' (Photobacterium fischeri* [ATCC 7744]*)::luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) *fusion; pACYC184*-derived, TetR, AHL biosensor producing bioluminescence in respond to short-chain AHL	[34]
Escherichia coli [pSB1075]	*lasR lasl' (P. aeruginosa PAO1)::luxCDABE (P. luminescens* [ATCC 29999]) fusion in pUC18 AmpR, AHL biosensor producing bioluminescence in respond to long-chain AHL	[34]

with gold, and observed under an SEM (JOEL JSM-6400, Japan).

Biosensors and Growth Conditions

Biosensors used are listed in Table 1. Culture of the biosensors was in Luria Bertani (LB) broth with shaking at 220 rpm. Routine culturing at 37°C supplemented with antibiotics was performed.

Anti-QS Assay: Quantification of Bioluminescence from *E. coli* [pSB401] and *E. coli* [pSB1075]

Production of bioluminescence was quantified using a luminometer (Infinite M200Pro, Switzerland). Briefly, an overnight culture of *E. coli* biosensors cells was diluted to an OD_{600} of 0.1 and treated with essential oils, and 200 µl was added into a 96-well Greiner microtiter plate. For *E. coli* [pSB401] and *E. coli* [pSB1075], *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL, 0.005 µg/ml) and 3-oxo-C12-HSL (0.1 µg/ml) were supplemented, respectively. Relative light units (RLU) per unit of optical density indicate production of bioluminescence at 495 nm, accounting for the influence of increased growth on the total bioluminescence [35]. Reduction of bioluminescence in *E. coli* [pSB401] *and E. coli* [pSB1075] suggested anti-QS properties of the essential oil. Biosensor cells treated with Tween 80 alone were used as the negative control.

Table 2. Chemical composition of *Cinnamomum verum* (cort.) essential oil.

Peak	Library/ID	RT	Area %	CAS No.
1	Cineole	10.4189	0.615	000470-82-6
2	Benzyl alcohol	10.8139	12.5037	000100-51-6
3	Linalool	13.174	1.3701	000078-70-6
4	α-Terpineol	16.5779	1.6252	000098-55-5
5	γ-Terpineol	16.8036	0.2485	000586-81-2
6	Cinnamaldehyde	17.5934	0.3181	000104-55-2
7	*trans*-Cinnamaldehyde	20.1417	72.812	014371-10-9
8	Eugenol	22.5677	6.5712	000097-53-0
9	L-Caryophyllene	24.4388	0.7485	000087-44-5
10	Cinnamoyl chloride	24.6081	0.2335	000102-92-1
11	Coumarin	25.0218	1.7618	000091-64-5
12	2-Methyl-1-naphthol	28.8771	0.7566	007469-77-4
13	Benzyl benzoate	34.3685	0.4359	000120-51-4

Library/ID – Identification of the compounds based on NIST library.
RT – retention time of the peak (scale).
Area% - % of peak area.

Statistical Analysis

Results shown represent the average readings of three independent experiments. Readings were presented as the mean ± standard deviation (SD). One-way analysis of variance and Student's *t*-test were used for the analysis. $P < 0.05$, indicating statistical significance, was calculated using the GraphPad Prism 5 statistical software.

Results

Gas Chromatography-Mass Spectrometry Analysis

The chemical composition study of *Cinnamomum verum* (cort.) oil elucidated by GC-MS is shown in Table 2. The major constituents (>1.0%) of the oil were *trans*-cinnamaldehyde (72.81%), benzyl alcohol (12.5%), eugenol (6.57%), coumarin (1.76%), α-terpineol (1.62%), and linalool (1.37%).

Time-Kill Assay

Synergistic interaction was observed between CB and piperacillin in the time-kill study (Fig. 1). The time-kill assay for synergy required a ≥2 $\log_{10}$ decrease in CFU/ml by the treatment for definition of synergism in comparison with the most active single drug and a ≥2 $\log_{10}$ decrease in the CFU/ml below the starting inoculum [19]. Detection of synergy was observed at 20 h post-treatment when there was a more than 2 log factors reduction in the number of

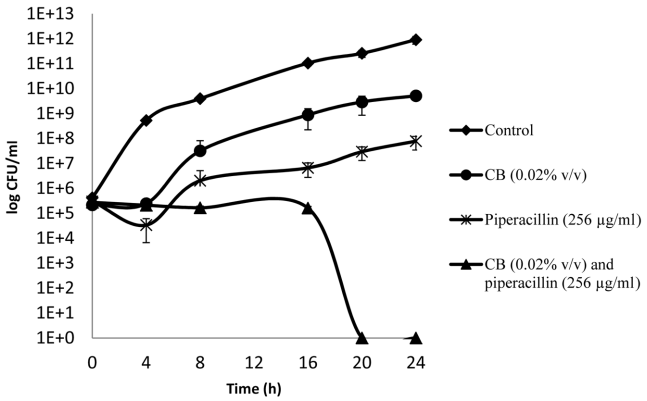

Fig. 1. Time-kill analysis of cinnamon bark oil (CB), piperacillin, or the combination of both against *E. coli* J53 R1.

Table 3. Reduction of membrane permeability of *E. coli* J53 R1 by cinnamon bark oil and/or piperacillin.

Treatment	OD_{625} ± SD (n = 3)				
Time (min)	Immediately	5	10	30	60
Control					
with 0.1% SDS	0.31 ± 0.003	0.32 ± 0.003	0.32 ± 0.006	0.32 ± 0.005	0.33 ± 0.005
without 0.1% SDS	0.31 ± 0.002	0.32 ± 0.003	0.31 ± 0.005	0.32 ± 0.007	0.33 ± 0.015
Cinnamon bark (0.02% (v/v))					
with 0.1% SDS	0.28 ± 0.002[a]	0.25 ± 0.002[b]	0.24 ± 0.002[b]	0.24 ± 0.007[b]	0.23 ± 0.002[b]
without 0.1% SDS	0.27 ± 0.001[a]	0.28 ± 0.002[b]	0.28 ± 0.006[b]	0.29 ± 0.004[b]	0.29 ± 0.003[b]
Piperacillin (256 µg/ml)					
with 0.1% SDS	0.31 ± 0.002	0.31 ± 0.003[b]	0.31 ± 0.002[b]	0.30 ± 0.002[b]	0.29 ± 0.007[b]
without 0.1% SDS	0.31 ± 0.002	0.31 ± 0.003[b]	0.32 ± 0.006[b]	0.32 ± 0.006[b]	0.32 ± 0.007[b]
Cinnamon bark (0.02% (v/v)) + Piperacillin (256 µg/ml)					
with 0.1% SDS	0.26 ± 0.002[a]	0.25 ± 0.002[b]	0.24 ± 0.003[b]	0.25 ± 0.004[b]	0.24 ± 0.007[b]
without 0.1% SDS	0.26 ± 0.002[a]	0.26 ± 0.002[b]	0.27 ± 0.015[b]	0.27 ± 0.005[b]	0.27 ± 0.004[b]

[a]Significant difference among treatment groups when compared with the corresponding control groups (with or without 0.1% SDS) ($p < 0.05$).

[b]Significant difference between samples treated and non-treated with 0.1% SDS at the corresponding time points ($p < 0.05$).

Values are the mean OD_{625} ± SD of three replicates.

viable cells by the drug combination when compared with CB treatment alone. Contrastingly, piperacillin alone at sub-concentration did not effectively reduce the cell numbers. CB alone at sub-concentration also did not show complete cell eradication as compared with the combination of CB and piperacillin.

Outer Membrane Permeability Test

SDS was used as a permeabilizing probe in this experiment. Table 3 shows the differences in absorbance between pre- and post-treatment of the control, essential oil, or/and antibiotic with or without 0.1% SDS.

Bacterial Surface Charge – Zeta Potential Measurement

The untreated *E. coli* J53 R1 tested had an original negative surface charge of -15 mV. All treatments in the study showed a reduction in the negative charges on the cell surface (Fig. 2).

Scanning Electron Microscopy

Electron microscope observations further illustrated the ultrastructural damage of *E. coli* after treatment with the essential oil and antibiotic combination (Fig. 3).

Anti-QS Assay: Quantification of Bioluminescence from *E. coli* [pSB401] and *E. coli* [pSB1075]

An *Escherichia coli* biosensor that will show a reduction in bioluminescence in the presence of QS inhibitors was exploited to confirm the possible anti-QS effect of the essential oil. The essential oil was verified to not produce a bactericidal effect on all biosensor cells, as determined by growth curve (Fig. 4). This experiment showed that concentrations of CB from 0.01% to 0.0075% to 0.005% showed inhibition of bioluminescence produced by *E. coli* [pSB401] and [pSB1075] (Fig. 5). No antimicrobial effects were observed in the performed assays when these concentrations were applied.

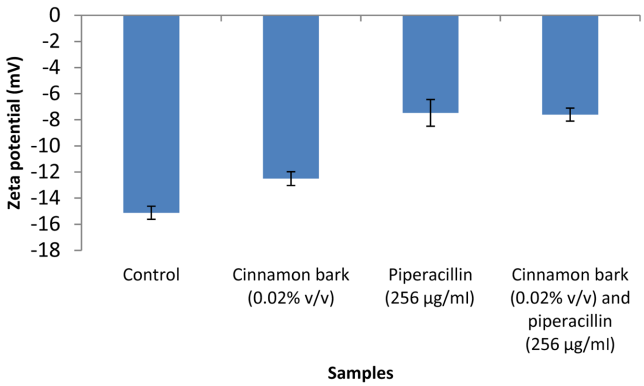

Fig. 2. Readings of zeta potential values (mV) of *E. coli* J53 R1 when exposed to cinnamon bark oil or/and piperacillin.
The mean ± SD for three replicates is indicated.

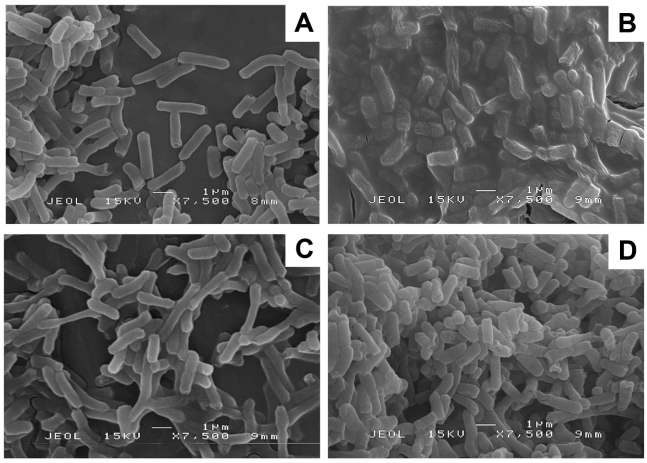

Fig. 3. *E. coli* J53 R1 under scanning electron microscopy.
(**A**) Treatment-free cells (control), (**B**) cinnamon bark (0.02% (v/v)), (**C**) piperacillin (256 μg/ml), and (**D**) cinnamon bark (0.02% (v/v)) plus piperacillin (256 μg/ml).

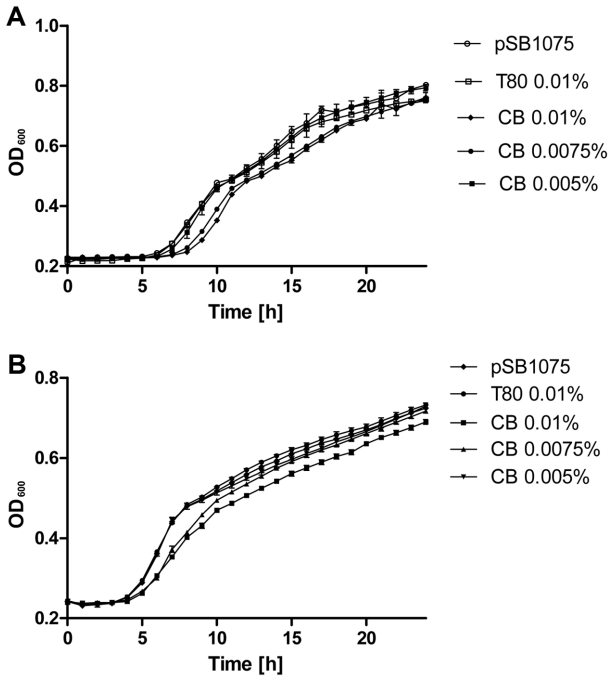

Fig. 4. Growth effect of CB at various concentrations on (**A**) *E. coli* [pSB401] and (**B**) *E. coli* [pSB1075].
Tween 80 was used as the negative control.

Discussion

Table 2 shows that a total of 13 compounds were identified. The predominant compounds present were *trans*-cinnamaldehyde (72.81%), benzyl alcohol (12.5%), and eugenol (6.57%). Similarly, previous studies conducted

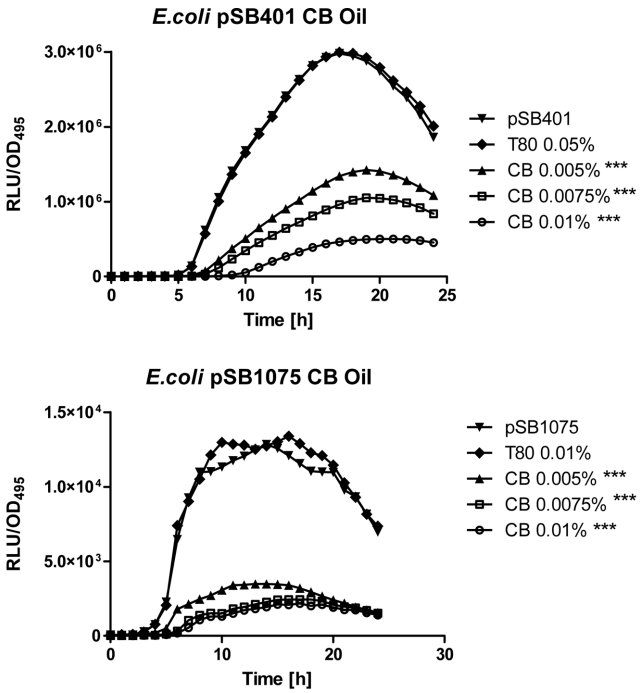

Fig. 5. Bioluminescence expression of *E. coli* [pSB401] and *E. coli* [pSB1075] by CB with concentrations from 0.01% (circle) to 0.075% (square) to 0.005% (triangle), while Tween 80 (diamond) and *E. coli* [pSB401/1075] supplemented with C6-HSL/ 3-oxo-C12-HSL, respectively, served as controls (inverted triangle).
The readings were presented as RLU/OD to account for any changes in growth. Data were analyzed by one-way analysis of variance with *** $p < 0.05$ being significant.

in South China on three species of cinnamon bark oils showed the presence of *trans*-cinnamaldehyde (66.28–81.97%) [17].

In gram-negative bacteria, beta-lactamases are secreted into the periplasmic space and act in combination with increased membrane permeability [12]. High-level antibiotic resistance is generally not attributed to the bacterial intrinsic resistance alone; it is attributed to a synergistic relationship between both the impermeability of the outer membrane and other extrinsic resistance such as enzymatic inactivation of antibiotics [12, 24]. Beta-lactam antibiotics have been known to target the cell membrane of bacteria through action on different targets, such as on the transpeptidases, transglycosylases, and carboxypeptidase [33]. To improve the efficacy of antibiotics, it is necessary to explore methods and study the modes of action that improve the diffusion of antibiotics and bypass the bacterial membrane barrier, which is responsible for the

general antibiotic resistance in gram-negative bacteria [4]. In this study, the mode of action of piperacillin was further expanded by the combination with CB, and resulted in potentiating the activity of the said antibiotic. Previous findings proving the gross and irreversible bacterial membrane disruption by natural product-antibiotic treatment have garnered support [8, 15].

In gram-negative bacteria, the hydrophilic porins are responsible for regulating the molecules passing through the outer membrane, based on their chemical nature. Exclusion of hydrophobic substances is often occurring outside the cells, possibly causing the weakening of the outer membrane by molecules that disintegrate the lipopolysaccharides layer; these agents are usually known as membrane permeabilizers [5, 31]. The results from the OM permeability test suggests that the outer membrane barrier is disturbed by the presence of the essential oils, and this phenomenon significantly enhances the effects of the antibiotics to bind to the penicillin-binding proteins (PBPs) that are localized on the outer face of the cytoplasmic membrane [12, 20]. Literature by Kojima and Nikaido [16] demonstrated that the influx of penicillin into *E. coli* occurs by a spontaneous passive and nonspecific diffusion through the porin channels. Sublethal concentrations of piperacillin alone did not increase outer membrane permeability, although influx of SDS occurred in the cells, and thus the OD readings were not decreased significantly ($p > 0.05$) throughout the course of this experiment owing to the hydrophilic nature of the beta-lactam antibiotics [18]. Sublethal injury of bacterial cell membranes may consequently disrupt the cell permeability and thus affect the membrane's ability to osmoregulate the cell adequately [13]. SDS readily dissolves the cytoplasmic membrane of bacteria owing to its chemical nature, but the treatment of *E. coli* with 0.1% SDS did not result in any significant lytic effect, as evidenced by the control without pre-treatment of antibiotic or CB. However, the duration of this experiment was limited to 60 min to prevent possible cell lytic reaction as a result of prolonged exposure to SDS [36]. CB at its subinhibitory concentration sensitized the bacteria against SDS. These findings are in substantial agreement with those studies carried out in eugenol, one of the major components found in cinnamon bark essential oils, whereby eugenol plays a role in disrupting the bacterial cell membrane without exerting bactericidal effects itself through enhancing the activities of the membrane permeabilizers such as lysozyme, Triton X-100, and SDS [15].

Other cellular structures may also be affected in a cascade type of action due to cell membrane disruption, thereby causing the bacterial cell wall lysis, followed by loss of intracellular dense material [7]. The scanning electron micrograph confirms this hypothesis when the overall bacterial cell surface treated with CB was considerably structurally disparate from the untreated cells. This lethal effect could be due to the disruption of the membrane structure, and it is supported by the previous work by other groups [2, 26, 28]. The rapid action of the combination pair observed in the time-kill analysis indicated treatment-induced gross cell damage. Cells treated with CB alone showed a corrugated surface, whereas combination with piperacillin showed gross cell damage with changes of size and shape. Scanning electron microscopy, however, is only able to provide a qualitative observation [6]. It does not provide a measurement on the magnitude of changes compared with the control; rather, it is evidence of physical disruption to the physiological state of the cell.

Details on the membrane potential and reflections on the bacterial metabolic state can be ascertained using the zeta potential measurement, whereby higher growth rates produce a more negative reading [30, 32]. Under normal physiological circumstances, bacterial cell surfaces are usually negatively charged; this is due to the presence of anionic groups in their membranes. However, there remains interspecies variance in the magnitude of the charge, possibly subjected to external influences. These include culture conditions such as pH and ionic strength [13, 25]. Our work demonstrated that the cells carried a reduced negative charge after exposure to CB. It is suggested that the irreversible membrane damage was caused by the acidifying and protein denaturation of the cell membrane due to the accumulation of the components of the essential oil [5]. Membrane damage allows access of antibiotics to the PBPs [4], and this hypothesis is supplemented by evidence of the OM permeability test in this study. It was suggested that the decrease of energy charge and low energy status are indications of a loss of metabolic energy. [29].

The current study also suggests the anti-quorum sensing ability of CB, where it showed promising inhibitory properties for long- and short-chain AHL quorum sensing systems. The QS mechanism is important in regulating bacterial behaviors in response to fluctuations in cell population, using chemical signals. This unique mechanism of bacterial community makes anti-QS a promising prospect to overcome bacterial pathogenicity without posing any selective pressure for the introduction of resistance [38]. In this study, the inhibitory effects were greatly increased in *E. coli* [pSB1075] and *E. coli* [pSB401] when the concentration

of the oil increased. Inhibition of *E. coli* [pSB1075] and *E. coli* [pSB401] which carry the *lasR* and *luxR* receptor gene, respectively, suggests the presence of anti-QS activity.

In the current study, the proposed main mode of action of CB is through the disruption of the bacterial membrane both at lethal and sublethal concentrations, subsequently increasing the nonspecific mobility of the antibiotic into the bacterial cell [10, 11]. These results indicated that CB not only has membrane permeabilizing activity but also anti-quorum sensing effects. Considering the heterogeneous composition of CB, it is highly likely that the mode of action of CB is more complex than the work demonstrated here. It is in line with this point of view that further research is needed to understand the mechanisms involved. This is vital to justify the practical applications of CB for use as a therapeutic option alongside current frontline antibiotics.

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