Chapter 15

Essential Oils: The Ultimate Solution to Antimicrobial Resistance in *Escherichia coli*?

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Additional information is available at the end of the chapter

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Abstract

Antimicrobial resistance (AMR) is on the rise; the only solution for overcoming this is through accelerated drug discovery. At current, bacterial evolutionary rates is still clearly the undisputed winner in this war. To circumvent this, evolution of resistance need to be curbed and this can only be effective via novel approaches, one of which includes the use of a resistance modifying agent. The criterion to qualify as a resistance modifier necessitates the co-administration of the agent with an inhibitor that deactivates the bacterial resistance mechanism, restoring its original effectiveness. Natural products such as plant extracts and essential oils (EOs) have been viewed as a privileged group for investigation of their potential roles to combat antibiotic resistance, due to their compositions of active chemical compounds. The route for multidrug resistance development in Gram-negative bacteria is primarily mediated by the sophisticated inner and outer membrane barriers, which function to protect the cell against external toxic compounds; hence, bypass of these bacterial membranes would successfully restore or improve efficacy of the antimicrobials. The aim of this chapter is to concisely describe some examples for recent strategies used in the screening of possible resistance modifiers from essential oils specifically against MDR *Escherichia coli*.

Keywords: essential oils, combination therapy, drug synergism, resistance modifying, membrane permeability

1. Introduction

Antimicrobial resistance is an especially pressing problem in the clinical setting today. The pinnacle of secondary infections due to convergence between communicable disease

(CD) and noncommunicable diseases (NCD) further complicates the problem [1]. There is a two-sided role for antibiotics; and although their uncontested and unquestionable role was recognized to significantly reduce the statistics of the infectious diseases burden worldwide, their rampant use also contributed to the unexpected emergence of antibiotic resistant microorganisms attributed to over-prescription and misuse, hence, the emergence of the multidrug resistant Enterobacteriaceae, especially *Escherichia coli* (*E. coli*). Adversely, the last line of antibiotics, colistin, which had only been recently revived since 1959 amidst the fairly new emergence of carbapenem-resistant *Enterobacteriaceae*, had been reported by Chinese researchers to be inefficacious against *E. coli* recently, in infected pigs from a farm near Shanghai, and the spread of colistin resistance had increased significantly especially in the agriculture industry over time, which may be escalated to a global scale [2, 3]. With the establishment of new resistance, the Chinese authors have emphasized the urgent need for coordinated global action in the fight against pan-drug-resistant Gram-negative bacteria and one of these strategies proposed included investigation into natural products, in this case, essential oils. This chapter aims to introduce the usage of synergistic combinatorial therapy between different classes of antibiotics and essential oils against multidrug resistant *E. coli* (MDR *E. coli*) and to detail the methodologies used to establish synergism as well as the mechanisms involved.

2. Antibiotic classes and multidrug-resistant *E. coli*

2.1. Antibiotic classes and their respective natures

From the discovery of penicillin by Alexander Fleming in the early nineteenth century, approximately 20 classes of antibiotic have been discovered in time. However, only antibiotic classes that are effective against *E. coli* would be thoroughly discussed in this subsection.

2.1.1. β-lactam antibiotics

β-Lactam antibiotics are one of the most common yet diverse classes of antibiotics and are the first class of antibiotics discovered in the 1930s. They were effective against both Gram-positive and Gram-negative bacteria and were categorized into four main groups, carbapenems, cephalosporins, monobactams, and penicillins, with each group sharing structural similarity in the β-lactam ring within the antibiotic molecule [4]. The β-lactam antibiotics mainly target the bacterial cell wall synthesis pathway, and are thus termed "broad spectrum antibiotics." Under normal physiological conditions, bacteria constantly renew their cell wall in order to replace broken ones. A unit of peptidoglycan cell wall consists of two subunits, the alternating *N*-acetylglucosamine and *N*-acetylmuramic acid. Each subunit contains an identical pentapeptide chain, which links both subunits together via the action of a transpeptidase, penicillin-binding protein (PBP) [5, 6]. β-Lactam antibiotics would act as an irreversible inhibitor toward PBP. The β-lactam ring of the antibiotic mimics the structure of the pentapeptide chain and thus is able to bind with PBP, acylating

it's active site and rendering it inactive [7, 8]. Hence, the action of β-lactam antibiotic halts cell wall synthesis of bacteria, which eventually compromises the rigidity of the cell wall, leading to cell lysis.

2.1.2. Fluoroquinolone

Fluoroquinolones is another class of antibiotics that exert their effect on both Gram-positive and -negative bacteria. The main structural feature of this particular antibiotic class is the presence of the fluorine atom within these antibiotics. It exhibits a broad spectrum activity against a large panel of bacteria as this group of antibiotic inhibits DNA synthesis by locking both the DNA gyrase and topoisomerase IV with the DNA strand during DNA replication. This prevents the action of other enzymes such as the RNA polymerase and DNA helicase for normal DNA replication, which eventually leads to cell death [9, 10]. Commonly prescribed fluoroquinolones include ciprofloxacin, gemifloxacin, levofloxacin, and moxifloxacin, which had relatively low adverse effects.

2.1.3. Aminoglycosides

Aminoglycoside is another major group of antibiotics showing enhanced potency toward Gram-negative bacteria. As the name suggests, this compound comprises of sugar units bounded to an amino group. Aminoglycosides exhibit high potency as well as a broad spectrum of action as it disrupts protein synthesis by binding only to the prokaryotic 30S ribosomal subunit, which then impairs the proofreading mechanism during protein translation [11, 12]. This disruption produces dysfunctional proteins, either due to misreading or premature termination, and eventually causes cell death. Even though aminoglycosides are specific toward prokaryotic ribosome, toxicity had been observed and reported in mammalian cells when a high dosage was applied [13]. Hence, aminoglycosides are only prescribed during life-threatening infections. Commonly prescribed aminoglycosides includes amikacin, gentamicin, and streptomycin.

2.1.4. Nitrofurans

Nitrofurans are a highly potent antibiotic class, which contain a furan ring and a nitro group. They are only used against urinary tract infections, especially when the infection is caused by an antibiotic-resistant pathogen. This is due to the high metabolism rate of the liver in partially breaking down the ingested nitrofuran. The remaining nitrofuran is then concentrated in the urinary bladder and thus suitable to be used in urinary tract infection, enabling targeted delivery [14]. High potency of nitrofuran is contributed by its diverse mode of actions when used against bacteria. In the presence of bacterial nitroreductases, nitrofuran is converted into reactive intermediates such as peroxynitrite and nitric oxide, which attack the bacterial ribosome, thus halting the protein synthesis in bacteria [15]. It was also reported that these reactive intermediates of nitrofuran can attack bacterial DNA as well as acting as a quorum sensing inhibitor [16, 17]. Due to the attribute of their multiple-action mode, resistance toward nitrofurans

has yet to be observed in pathogens. Nonetheless, the exact mechanism of nitrofuran has yet to be fully understood. Nitrofurantoin is the common form of nitrofuran, which is prescribed generally.

2.1.5. Polymyxin

Polymyxin is a lipopeptide antibiotic that had been sidelined previously due to its high toxicity against mammalian cells. However, the emergence of multidrug-resistant pathogens has caused a resurgence in the use of polymyxin in treatments for bacterial infections as last resort. Polymyxin consists of a cyclic peptide bounded to a long hydrophobic fatty acid tail and it targets mainly Gram-negative bacteria [18, 19]. Potency is only targeted toward Gram-negative bacteria due to their mode of action, whereby the fatty acid tail of the antibiotic specifically targets and binds to the lipid moiety of a lipopolysaccharide, Lipid A that can only be found in Gram-negative bacteria. This results in the insertion the cyclic peptide of the antibiotic into the cell membrane, thus compromising its integrity and increasing the permeability of the cell membrane. This eventually causes cytoplasmic leakage and leads to cell death [20–22]. Commonly prescribed polymyxin includes colistin and Polymyxin B.

2.2. Antibiotic resistance mechanisms in MDR *E. coli*

The introduction of antibiotics as therapeutic agents to treat bacterial infection or as a growth promoter in molecular engineering had adversely propelled bacterial evolution, forcing bacteria to develop resistance mechanisms in order to survive within an antibiotic-filled environment. This gave rise to multidrug-resistant (MDR) pathogens, especially *E. coli* as they are commensal microorganisms and often used as the model bacteria in research. The emergence of MDR *E. coli* has posed a great threat toward the survivability of mankind, thus, the in-depth understanding of the strategies used by MDR *E. coli* to bypass antibiotic treatment is necessary to address this issue.

MDR *E. coli* exhibits the ability to resist multiple antibiotics simultaneously due to the acquisition of several genes that confer abilities such as antibiotic inactivation, multidrug efflux pump, target modification, or overproduction and reduction of cell membrane permeability. The multidrug efflux pumps are energy-dependent and have been reported to be overexpressed in the presence of antibiotics, helping it to expel antibiotics that had successfully permeated into the cell [23]. The multidrug efflux pumps indicated low specificity enabling the removal of antibiotics beyond the same class, rendering the antibiotics ineffective. For instance, efflux pump AcrAB-TolC of RND family is able to expel β-lactam antibiotic, fluoroquinolones, tetracycline, and glycylcycline [23–26]. Furthermore, MDR *E. coli* can alter their outer membrane permeability by modifying the structure of porins and/or reduce or stop their expression, which would be ultimately responsible for antibiotic access into the cell [27]. It has been reported that porins observed in MDR *E. coli* had narrower channels when compared to normal strains, which prevents the antibiotics from entering the cell [28]. MDR *E. coli* had been reported to be able to deactivate antibiotic with the production of antibiotic-targeting enzymes. β-Lactamase is one example of enzymes produced by MDR

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E. coli, which has the ability to cleave β-lactam antibiotic, rendering it nonfunctional [29]. Antibiotic target modification had also been observed in MDR *E. coli*. Penicillin-binding protein (PBP), a transpeptidase, which links peptidoglycan subunit together, is the main target of the β-lactam antibiotic. It has been observed that isolated PBP from MDR *E. coli* had conformational differences when compared to nonresistant strains of *E. coli*. This slight conformational change prevents effective binding of β-lactam antibiotic but allows the transpeptidase to carry out its normal physiological function [30].

3. Synergistic potential of essential oils and antibiotics: challenges

The emergence of multidrug-resistant pathogens, especially *E. coli*, have caused an interest shift from the onerous development of novel classes of antibiotics to the more straightforward application of synergism or combinatory therapy in the hope of reviving the efficacy and effectiveness of existing antibiotics. Quite a number of publications regarding the usage of essential oils and antibiotics as a combinatory therapy have indicated great success, with significant reductions in the dosage of antibiotics required to completely annihilate multidrug-resistant pathogens [31–36]. Despite this, the usage of essential oils as a component for combinatory treatment posed a few challenges in its application. For instance, solubility of the hydrophobic essential oil in the aqueous medium is one of the greatest challenges faced. To solve this problem, emulsifiers such as dimethyl sulfoxide (DMSO) and polysorbate 80 (Tween 80) had been used to increase the solubility of essential oils in the aqueous medium. This would ensure maximum contact between the test organism as well as the test compound used throughout the experiment [37]. The concentration of such emulsifiers should also be taken into consideration as high concentration would cause toxicity to the test organism, resulting in false positivity during testing. For example, usage of DMSO at a concentration of more than 4% would reduce the viability of *Salmonella paratyphi* A, *Staphylococcus epidermis*, *Shigella flexneri*, *Vibrio cholerae,* and *Pseudomonas oleovorans* to less than 50% [38]. To better address the solubility issue, there is need to standardize the method used to determine synergism. The broth microdilution method has been shown to be the most accurate when compared to other susceptibility tests such as the disk diffusion and agar dilution methods, which are less informative [39]. In order to further maximize solubility, the incubation parameter should be standardized to shake at 200 rpm to ensure the formation of consistent emulsion, a crucial attribute in indicating the solubility of essential oils.

Another challenge faced when using essential oils in combinatorial therapy would be the volatility of essential oils. It has been well documented that essential oils consist of 20–60 compounds, which are highly volatile, but none of which are actually lipid in nature [40]. Thus, with the solubility problem solved, volatility of essential oils is the next problem to tackle in order to achieve accurate determination of synergism in combinatorial therapy. Volatility of essential oils can be affected by several factors. For instance, exposure to light can accelerate the degradation as well as volatility of essential oils. It has been demonstrated that in the presence of light, the autoxidation process of essential oil was accelerated, leading to the loss

of several compounds within the essential oil itself [41, 42]. Another factor that can affect the volatility of essential oils would be the temperature. As temperatures increases, the autoxidation and degradation process of essential oils are markedly increased [43]. However, little can be done about the temperature factor as heat is still required for the test organism to grow optimally. At the least, testing should be carried out with minimal light to reduce the autoxidation and degradation of the essential oils.

4. Establishment of synergism

In combination therapy, synergy is said to occur when the combined effect of two agents is greater than the sum of the individual effects. Currently there is no clear standardization or regulation of the methodology in combination therapy [44], further complicated by different test methods, different EOs extraction methods and test assays. The most widely used techniques to detect synergy are the checkerboard and time-kill curve methods [33, 45–48]. In checkerboard assay, in which two test agents are tested individually in serial dilutions and in all combinations of these dilutions together to find the concentration of each test agent, both alone and in combination, that produce some specific antimicrobial effects i.e., minimal inhibitory concentration (MIC). In antibiotics and EOs synergistic testing, the combined effects of the antibiotics and EOs are calculated and expressed in its fractional inhibitory concentration (FIC) using the following formula:

$$\text{FIC} = \frac{\text{MIC of EOs or antibiotic in combination}}{\text{MIC of EO or antibiotic alone}} \quad (1)$$

The sum of these fractions is expressed as fractional inhibitory concentration index (FICI) where:

$$\text{FICI} = \text{FIC of EO} + \text{FIC of antibiotic} \quad (2)$$

When FICI is less than or equal to 0.5, the combination is said to be synergistic; when FICI is between 0.5 and 4.0, the combination is said to have no interaction while FICI is more than 4.0, the combination is antagonistic [49]. Although checkerboard assay is by far one of the most reliable methods for demonstrating synergy, culture conditions predominantly influence the outcome of the study hence determinant factors should be precisely reported in manuscripts to better facilitate reproducibility of these experiments.

4.1. Investigations into membrane-specific effects in combination therapy

Bacterial peptidoglycan/ cell wall disruption remains one of the most promising approaches for EO-mediated cell death. Numerous data are already available on membrane disruptive effects of EOs against the Gram-negative bacteria including *E. coli* [50–54]. In our previous work, several encouraging synergistic combinations of EOs and antibiotics against beta-lactam resistant *E. coli* were obtained. Our understanding of how EOs synergies antibiotic action and induce bacterial cell death is focused on the generalized membrane disruptive effects of the EOs.

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4.1.1. Assessing bacterial surface charge using zeta potential measurement

The zeta potential is a consequence of the existence of surface charge; it provides the information on the electrophoretic mobility of the dispersed particles. Zeta potential measurement can be used to investigate the membrane potential, which reflects the inherent metabolic state of the bacteria. Zeta potential reflects the electrical potential interface between the aqueous solution and the layer of such fluid attached to the bacterial cell, suggesting that loss of bacterial cell charge is related to the metabolic energy loss [55]. It has been found that the values are more negative at higher growth rates [56, 57]. The bacterial cell surfaces are negatively charged under normal physiological conditions, owing to the presence of anionic groups such as carboxyl and phosphate in their membranes. The magnitude of the charge varies between species and it fluctuates in response to various culture conditions such as the pH and ionic strength of the culture [58, 59]. More recently, we have employed technology using a Nano Zetasizer (Malvern Instruments, UK) to investigate the influence of antibiotic-EO combinations on the cell surface physiology of *E. coli*. Different concentrations of piperacillin exerted different degree of zeta potential reduction in *E. coli* J53 R1. It has been observed that when the concentration of the antibiotic increased, the cells became less negatively charged (**Figure 1**). The cells' zeta potential also responded differently to different types of EOs treatments at different test concentrations (**Figure 2**). The technique of electrophoretic light scattering offers advantages on the study of membrane potential with accuracy, measurement time and ease of use [60]. The work of Halder et al. further validated the use of zeta potential measurement as a measurable variable for membrane permeability studies [61].

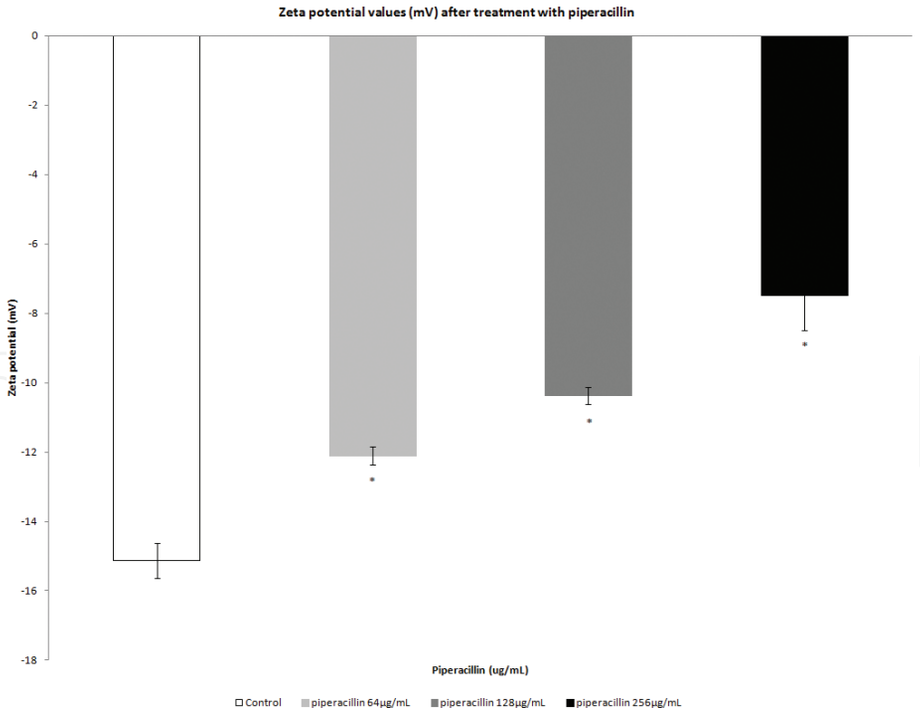

Figure 1. Zeta potential values (mV) of suspensions of *E. coli* J53 R1 when exposed to different concentrations of piperacillin treatments. File represents: (□) control; (□) piperacillin (64 µg/mL); (□) piperacillin (128 µg/mL); (■) piperacillin (256 µg/mL). The mean ± SD for three replicates is illustrated. Data were analyzed by one-way analysis of variance with $*P < 0.05$ being significant different from the control.

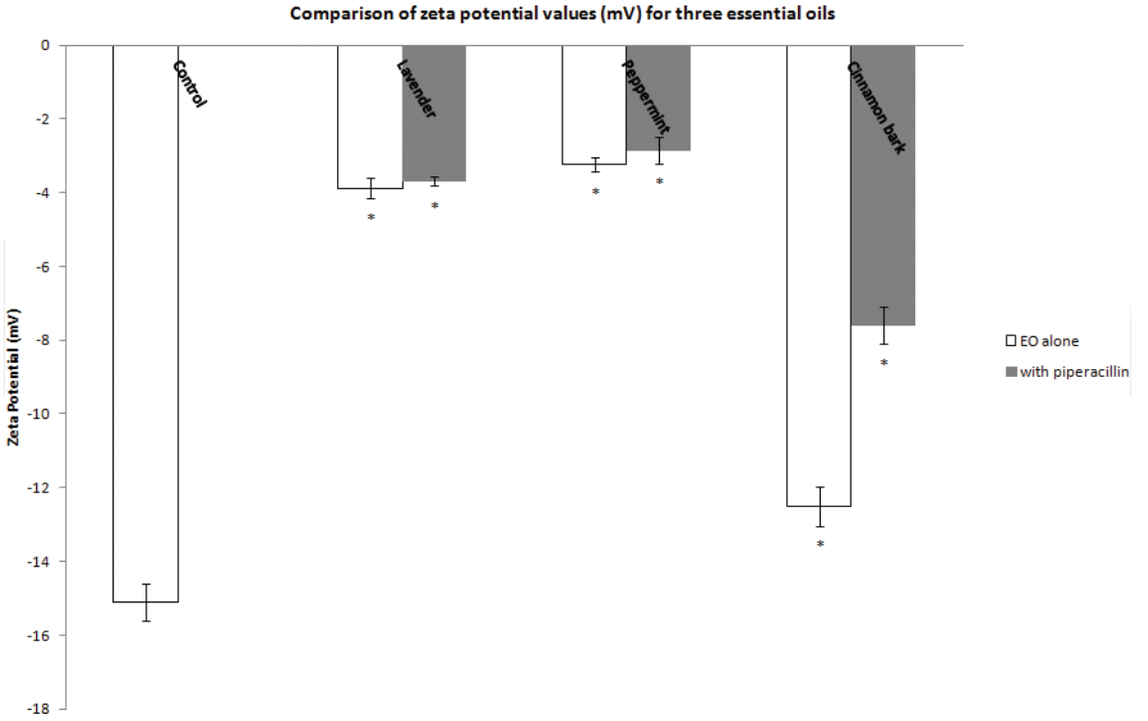

Figure 2. Zeta potential values (mV) of suspension *E. coli* J53 R1 when exposed to different EOs alone (□) or in combination with antibiotic (■). The mean ± SD for three replicates is illustrated. Data were analyzed by one-way analysis of variance with *$P < 0.05$ being significant different from the control.

4.1.2. Illustrations of cell physical changes using electron microscopy

In the study of membrane-active mechanisms, scanning electron microscope (SEM) is employed to directly observe cell morphological changes after treatments. In our work, we observed the morphological changes of *E. coli* after treatment with EOs, namely peppermint, lavender, and cinnamon bark. In the nontreated cells, a rod-shape morphology that is characteristic of *E. coli* was observed (**Figure 3**); and cells treated with beta-lactam antibiotic at different concentrations did not show any observable alterations in size, shape, and surface morphology (**Figure 4**). Interestingly, cells treated with cinnamon bark EO were observed to show surface irregularities and corrugation, as is similar to the cells treated with lavender and peppermint EOs (**Figure 5**). It is important to note that a disturbed cell membrane system would affect other cellular structures in a cascade type action. In addition to SEM, transmission electron microscope (TEM) is also often employed to study the membrane integrity and intracellular alteration of the bacterial cells before and after treatments.

4.2. Investigations on antiquorum sensing properties of EOs

N-acyl-L-homoserine lactone (AHL)-mediated quorum sensing is a widespread system of stimuli and responses, which regulates the virulent determinants in most Gram-negative bacteria [62]. Antiquorum sensing antimicrobials are unlikely to contribute to the development of

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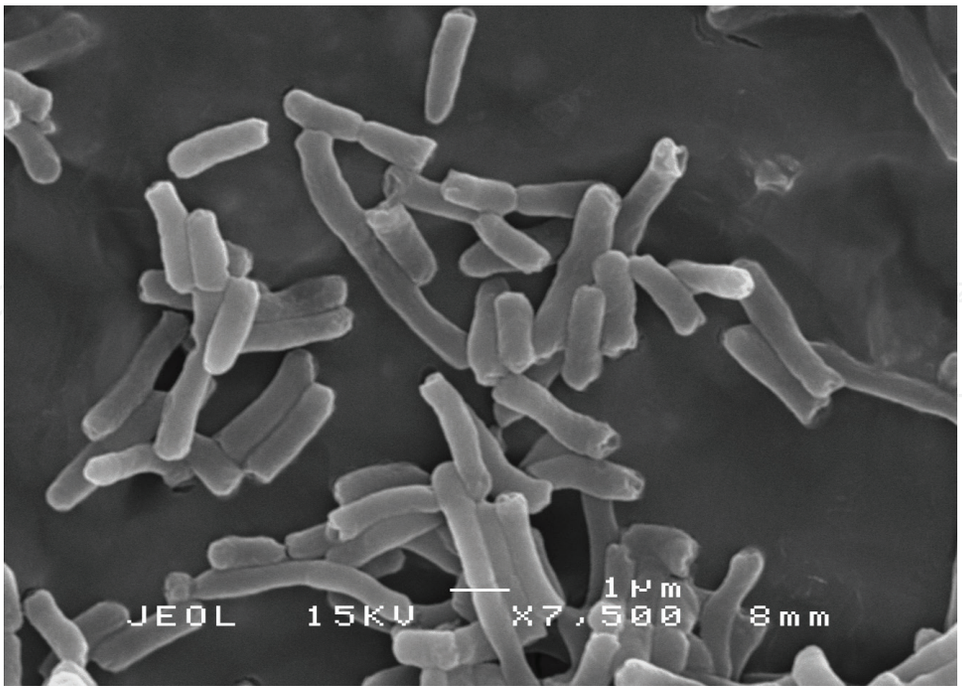

Figure 3. Scanning electron micrograph of the untreated *E. coli* J53 R1.

multidrug-resistant pathogens since it does not impose any selection pressure. Consequently, quorum sensing has been viewed as an attractive alternative strategy used to combat bacterial antibiotic resistance. The lack of AHL synthase-encoding gene, which should be naturally occurring of *E. coli* has made this variant a suitable biosensor for the screening of AHL synthase inhibitors. Experimentally, external AHLs are supplied exogenously to induce quantifiable quorum sensing traits such as bioluminescence. The antiquorum sensing ability of the test compounds are then measured by the significance of light inhibition [63]. In our previous work, we have employed *E. coli* [pSB401] and [pSB1075], which produce bioluminescence in response to short and long chain AHL respectively as the biosensors [64]. *Lavandula angustifolia* and *Cinnamomum verum* bark essential oils were found to significantly inhibit the light production of the biosensors, indicating the possibility of these EOs as quorum-sensing inhibitors [31, 32].

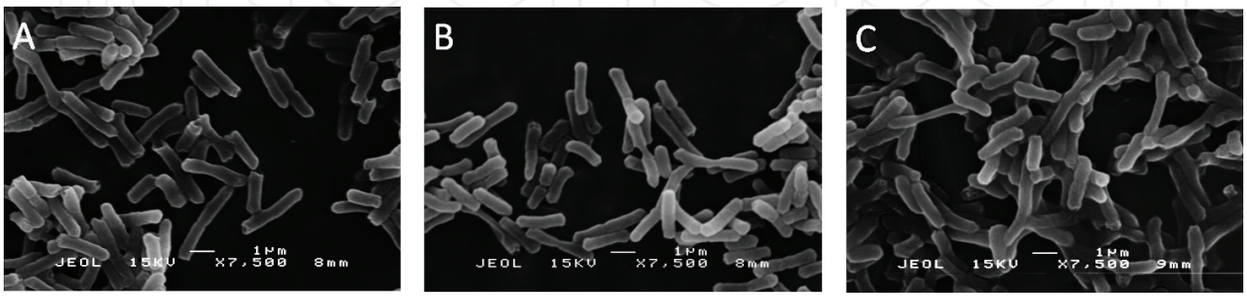

Figure 4. Scanning electron micrographs of *E. coli* J53 R1 after treatment of piperacillin at (a) 64 μg/mL, (b) 128 μg/mL, and (c) 256 μg/mL.

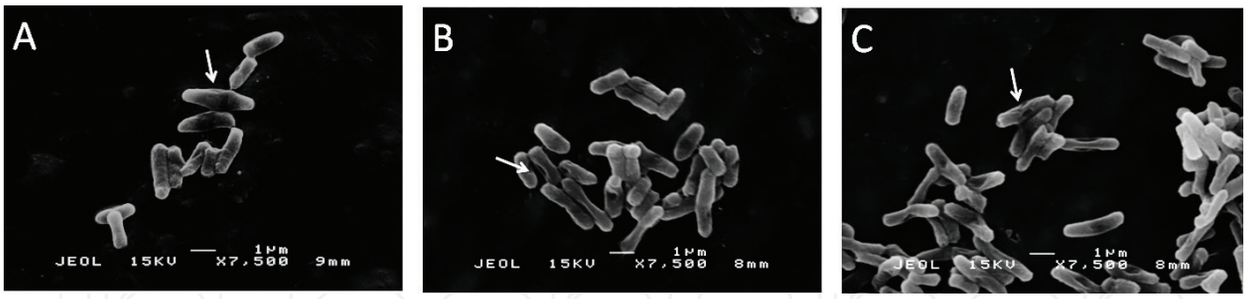

Figure 5. Scanning electron micrographs of *E. coli* J53 R1 after (a) peppermint, (b) lavender, and (c) cinnamon bark essential oils treatment.

5. Moving forward: present and future prospects

The exploitation of EOs has shed new light on antimicrobial therapeutics research and also the resurgence in the use of herbal medicine worldwide. Although possibilities of combination therapy appear to be extensive, the mode of interaction between two antimicrobials is extremely crucial. One of the challenges encountered in the *in vitro* study on a particular antibiotic is that despite proven synergism, it does not guarantee the success of the clinical use of the therapeutic agent. A major issue to be addressed is the pharmacology aspects of the membrane active properties of the EOs as a candidate therapeutic agent and their precise condition of use. Thus, in line with *in vitro* susceptibility testing, *in vivo* experiments are needed in tandem to provide sufficient supporting evidence to serve as a basis for new antimicrobials to survive through the phases of clinical trials.

In view of current efforts in developing alternative strategies by combining antibiotics with other compounds (antibiotic or nonantibiotic) —following the encouraging paradigm in Augmentin, this approach needs to be intensified. Besides inhibiting the effector molecules such as β-lactamase or DNA replication, supplementary compounds that interfere with regulatory mechanisms such as virulence genes or cell physiology have shown great potential. Furthermore, targeting nonessential bacterial pathways is also an alternative and very possible strategy employed to reduce the risk of developing resistance. Ultimately, just because bacteria can evolve in various ways to resist antibiotics at the rate that is insurmountable by new antibiotic development, it would be imperative for medical researchers to employ multiple strategies in the combat of antibiotic resistance. There is no single "magic bullet" to adequately address the phenomenon of multidrug resistance evolution.

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