

## Review

# Combination Therapy Involving *Lavandula angustifolia* and Its Derivatives in Exhibiting Antimicrobial Properties and Combatting Antimicrobial Resistance: Current Challenges and Future Prospects

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**Abstract:** Antimicrobial resistance (AMR) has been identified as one of the biggest health threats in the world. Current therapeutic options for common infections are markedly limited due to the emergence of multidrug resistant pathogens in the community and the hospitals. The role of different essential oils (EOs) and their derivatives in exhibiting antimicrobial properties has been widely elucidated with their respective mechanisms of action. Recently, there has been a heightened emphasis on lavender essential oil (LEO)'s antimicrobial properties and wound healing effects. However, to date, there has been no review published examining the antimicrobial benefits of lavender essential oil, specifically. Previous literature has shown that LEO and its constituents act synergistically with different antimicrobial agents to potentiate the antimicrobial activity. For the past decade, encapsulation of EOs with nanoparticles has been widely practiced due to increased antimicrobial effects and greater bioavailability as compared to non-encapsulated oils. Therefore, this review intends to provide an insight into the different aspects of antimicrobial activity exhibited by LEO and its constituents, discuss the synergistic effects displayed by combinatory therapy involving LEO, as well as to explore the significance of nano-encapsulation in boosting the antimicrobial effects of LEO; it is aimed that from the integration of these knowledge areas, combating AMR will be more than just a possibility.

**Keywords:** antimicrobial resistance; combination therapy; lavender essential oil; nanoencapsulation; synergy

## 1. Introduction

The phenomenon of antimicrobial resistance (AMR) has escalated substantially over the past few decades and it has been ascertained to be one of the greatest global health crisis at present [1]. AMR can be broadly categorized into three different patterns of resistance exhibited in AMR-organisms: multi-drug resistant (MDR) is defined as acquired non-susceptibility to one agent in at least three or more different antimicrobial categories, extensively-drug resistant (XDR) means non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (where bacterial isolates are susceptible to agents from only one or two antimicrobial categories), and pan-drug resistant (PDR) refers to non-susceptibility to all agents in all available antimicrobial categories [2]. Emergence of different strains of drug-resistant pathogens, especially in a significant proportion of hospital-acquired infections has rendered the use of conventional antimicrobial agents ineffective worldwide [3,4]. A specific group of MDR-organisms known as the “ESKAPEE” pathogens which encompasses seven different bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and

*Escherichia coli*, are the leading causes of hospital-acquired or nosocomial infections [5,6]. These pathogens are also associated with a significant risk of mortality and morbidity in hospitalized patients as a consequence of therapeutic failure, resulting in considerable healthcare and economic repercussions [7]. In the United Kingdom, incidences of bloodstream infection caused by MDR-pathogens, particularly Enterobacteriaceae like *K. pneumoniae*, *E. coli*, and Gram-positive bacteria like *Enterococcus* spp. in the hospitals have steadily increased by 32% between year 2015 and 2019 [8]. Similarly, in Malaysia, the most recent National Surveillance of Antibiotic Resistance report in 2016 has noted significant increase in prevalence of meropenem-resistant *Acinetobacter baumannii* and vancomycin-resistant *Enterococcus faecium* in various hospitals, resulting in poorer patient prognosis [9]. It has been postulated that the incessant dissemination of AMR is accelerated by many extrinsic and intrinsic factors. Inappropriate and indiscriminate prescribing of antibiotics in clinical settings due to non-adherence to proper antibiotic stewardship remains as the leading extrinsic cause of the emergence of AMR [10,11]. Other extrinsic factors such as widespread and unregulated use of antibiotics in veterinary and agricultural sectors, patient's non-adherence to prescribed antibiotics and unauthorized self-prescribing of easily-available antibiotics which are over-the-counter tended to speed up the trajectory of AMR [12,13].

On the other hand, intrinsic resistance in bacteria to antibiotics is acquired through inherent or mutational changes in functional or structural attributes of both the pathogens or molecular targets. These adaptations obtained by resistant strains of pathogens are mediated by genetic mutations in the bacteria themselves or through horizontal gene transfer. Certain bacteria possess the ability to employ certain hydrolytic enzymes to inhibit the intracellular binding between the drug and the target pathogen [14]. One such example would be the production of *K. pneumoniae* carbapenemase (KPC) seen in *K. pneumoniae* which degrades antibiotics, such as the  $\beta$ -lactam antibiotics (including carbapenems), aminoglycosides, and fluoroquinolones, before reaching the drug-binding protein targets, eventually nullifying their antimicrobial effects [15,16]. Another essential mechanism utilized by MDR-pathogens is via the presence of active drug efflux pumps, which promotes the active transport of the antibiotics out of the bacterial cell, eventually decreasing the intracellular concentration of the drug significantly [17,18]. Efflux pump up-regulation is more commonly seen in Gram-negative organisms, particularly in biofilm-producing *P. aeruginosa*, whereby the presence of different efflux pumps confers additional biofilm resistance to different forms of antibiotics [19].

Therefore, due to the rapid spread and acceleration of life-threatening MDR-strains of pathogens, there is a dire need in researching for novel yet effective antimicrobial agents and possible alternatives involving natural products to mitigate the development of AMR. Various natural compounds with medicinal properties have been proposed as antimicrobial agents against MDR pathogens, especially when used in association with conventional antibiotics [20,21]. Plant-derived metabolites such as essential oils (EOs) have been investigated extensively for their tremendous use as antimicrobial agents [22,23]. EOs are naturally-occurring compounds which are extracted from plants and they consist of different small complexes which are lipophilic and highly volatile [24,25]. In recent years, there has been a heightened emphasis on the therapeutic benefits of lavender essential oils (LEOs) and their derivatives especially on their antimicrobial effects. LEOs (primarily *Lavandula angustifolia*) have been shown to possess an extensive array of biological properties such as analgesic, antimutagenic, anti-inflammatory, anxiolytic, and a range of antimicrobial benefits [26–30]. Furthermore, combinatorial therapies incorporating the use of EOs has been shown by numerous in vitro studies to drastically potentiate the bactericidal effects against the MDR pathogens, which can be potential approaches in mitigating AMR [31,32]. Such strategies can be adopted via a few different combinations: (i) combination of different natural adjuvants (i.e., combining LEO with one or more types of EOs), (ii) incorporation of LEO into conventional antibiotics, (iii) optimization of LEO with inclusion of nanoparticles [33]. EOs (including LEOs) have poor oral bioavailability

and are chemically unstable to oxygen and humidity, which may limit their application as potential novel antimicrobial agents [34]. However, incorporation of LEOs into different types of nanoparticle delivery systems enables a more sustained and controlled release of the EOs, which enhances their antimicrobial benefits [35].

To date, there are limited reviews focusing on the antimicrobial benefits of LEOs and none elucidating the antimicrobial benefits of combinatorial therapies involving LEOs. This review aims to highlight the main antimicrobial properties that are exhibited by LEOs and their derivatives. In addition, application of different combinatorial therapies involving LEOs in augmenting their antimicrobial effects will be outlined, including the advantages of nano-based approaches in potentiating the therapeutic benefits of LEOs.

## 2. Components of LEO with Their Respective Antimicrobial Properties

Numerous qualitative and quantitative studies, done via different methods such as the gas chromatography, high-performance liquid chromatography and gas chromatography/mass spectrometry analyses have been conducted extensively in the past to identify the different constituents of LEOs [36,37]. Although there is a considerable amount of variation in terms of the chemical composition of different LEOs due to different areas of plant cultivation, presence of various plant genotypes and different oil extraction methods [38,39], it has been substantiated that it is the synergistic interaction from different components in the LEO that augments its antimicrobial effects.

By and large, LEO is primarily comprised of monoterpenes such as linalool, linalyl acetate,  $\beta$ -ocimene (both *cis*- and *trans*-) and lavandulol. Other sesquiterpenes-based compounds like  $\beta$ -caryophyllene and esters, such as lavandulyl acetate, can also be found in LEO [30,40]. Linalool and linalyl acetate constitute the highest proportion of chemical compounds found in extracted LEOs, with percentages ranging from 20 to 40% and 25 to 50%, respectively [41]. Table 1 illustrates the main terpene and terpenoid derivatives found abundantly in LEO that have been proven by previous studies to demonstrate promising antimicrobial properties.

**Table 1.** Relative abundance of main compounds found in lavender essential oil (LEO) and their antimicrobial effects from different quantitative and qualitative studies.

Chemical Components	Molecular Formula	Percentage (%)	Possible Mechanism of Action in Exhibiting Antimicrobial Effects	References
Linalool	C <sub>10</sub> H <sub>18</sub> O	20–40	Inhibition of bacterial growth. Disruption of cellular membrane.	[16,42]
Linalyl acetate	C <sub>12</sub> H <sub>20</sub> O <sub>2</sub>	25–50	Disruption of cellular membrane.	[43,44]
$\beta$ -ocimene	C <sub>10</sub> H <sub>16</sub>	3–5	Disruption of cellular membrane.	[45]
Terpinen-4-ol	C <sub>10</sub> H <sub>18</sub> O	3–8	Inhibition of bacterial growth. Disruption of cellular membrane. Inhibition of biofilm formation.	[46,47]
Eucalyptol (1,8-cineole)	C <sub>10</sub> H <sub>18</sub> O	1–4	Inhibition of bacterial growth. Disruption of cellular membrane. Inhibition of efflux pumps.	[48,49]
Camphor	C <sub>10</sub> H <sub>16</sub> O	1–10	Disruption of cellular membrane. Inhibition of biofilm formation.	[50,51]
$\beta$ -caryophyllene	C <sub>15</sub> H <sub>24</sub>	2–5	Disruption of cellular membrane.	[52]
Geraniol	C <sub>10</sub> H <sub>18</sub> O	2–5	Disruption of cellular membrane. Inhibition of biofilm formation.	[53,54]
Lavandulyl acetate	C <sub>12</sub> H <sub>20</sub> O <sub>2</sub>	3–8	Inhibition of bacterial growth.	[55]
Linalyl anthranilate	C <sub>7</sub> H <sub>23</sub> NO <sub>2</sub>	2–12	Disruption of cellular membrane.	[56]

As shown in Table 1, most of the chemical compounds found in LEO exhibits their antimicrobial effects by destroying the lipid cellular membrane of the pathogens, causing increased permeability to these compounds, leakage of intracellular molecules, and eventually irreversible cellular damage. This is possibly due to the fact that LEO and most of its constituents are lipophilic in nature, which promotes the penetration and accumulation of hydrophobic LEO into the phospholipid bilayer of the cellular membrane of the microbes [57].

In previous studies, oxygenated monoterpenes like eucalyptol, linalyl acetate, and linalool are associated with greater antimicrobial effects due to their lipophilic and/or hydrophobic properties [58,59]. Therefore, it is not surprising that LEOs have been proven to possess a broad spectrum of antimicrobial capacity against different Gram-positive and Gram-negative bacteria, a number of fungi such as yeasts and dermatophytes and as well as some parasites like *Schistosoma* spp. and *Trichomonas vaginalis*. The in vitro antimicrobial activities exhibited by LEOs are screened and assessed by measuring the diameters of zones of bacterial growth inhibition, determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration or minimum fungicidal concentration (MBC/MFC) levels against different pathogens. The MIC of LEO is defined as the lowest concentration of LEO required to inhibit the growth of microbial colonies tested. On the other hand, the MBC value of LEO denotes the minimum concentration needed to kill 99.9% or more of the pathogens, which is an indicator of LEO's bactericidal activity [60,61]. These parameters are evaluated using common bioassays such as the disc-diffusion method, broth macro- and microdilution assays [62]. However, the time-kill test has been found to be the best tool to ascertain the bactericidal or fungicidal effects of LEO due to its ability to establish the presence of any dynamic interaction between LEO and the microbes; which can be concentration-dependent or time-dependent [62,63]. Table 2 shows the MIC values of different clinically relevant pathogens that are obtained from a range of in vitro studies.

**Table 2.** Minimum inhibitory concentration (MIC) values of LEO against various pathogens obtained from different in vitro studies.

Pathogens	MIC Values (µg/mL)	References
<b>Gram-positive bacteria</b>		
<i>Staphylococcus aureus</i>	5.0	[33,64–67]
<i>Listeria monocytogenes</i>	5.5	
<i>Staphylococcus epidermidis</i>	4.0	
<i>Bacillus cereus</i>	25.0	
<i>Enterococcus faecalis</i>	1.3	
MRSA	100.0	
<b>Gram-negative bacteria</b>		
<i>Escherichia coli</i>	10,000.0	[68–71]
<i>Klebsiella pneumoniae</i>	10,000.0	
<i>Pseudomonas aeruginosa</i>	5000.0	
<i>Proteus mirabilis</i>	1000.0	
<i>Acinetobacter baumannii</i>	2000.0	
<b>Fungi</b>		
<i>Candida albicans</i>	10.0	[71–74]
<i>Trichophyton rubrum</i>	1.0	
<i>Trichosporon beigeli</i>	2.0	
<i>Cryptococcus neoformans</i>	1000.0	
<i>Aspergillus fumigatus</i>	3000.0	

The role of LEO as an alternative antimicrobial agent warrants special attention, particularly in clinical settings against MDR-bacteria as a few studies have reported its therapeutic benefits against different MDR pathogens like *A. baumannii* and *P. aeruginosa*. Sienkiewicz et al. (2014) conducted a study to evaluate the antibacterial properties exhibited by cinnamon, geranium, and LEOs against strains of *A. baumannii* isolated

from hospitals which are resistant to most conventional antibiotics, including trimethoprim/sulfamethoxazole, tobramycin, and tigecycline. MIC levels were determined using the broth microdilution method and all the EOs including LEOs have been shown to exhibit inhibitory activity against these resistant strains of *A. baumannii* [75]. In another study, Nikolic et al. (2014) evaluated the cytotoxic and antimicrobial effects of EOs from five different *Lamiaceae* species, including *L. angustifolia* through the microdilution method. Seven bacterial species consisting of *Streptococcus pyogenes*, *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Lactobacillus acidophilus*, *Enterococcus faecalis*, and *P. aeruginosa*, along with fifty-eight other clinical isolates of oral *Candida* spp. were used in the study. All the EOs, including *L. angustifolia*, have displayed significant bactericidal and fungicidal effects against all tested microbes [76]. On the other hand, Imane et al. (2017) studied the antimicrobial effects of LEOs against three of the most common causes of nosocomial skin and soft tissue infections: *S. aureus*, *P. aeruginosa*, and *E. coli*. From the disc diffusion test, it was reported that LEO exhibited bactericidal effects against both *E. coli* and *P. aeruginosa* with MBC values of 10.67 and 85.33  $\mu\text{L/mL}$ , respectively [77]. Furthermore, the antimicrobial potential and cytotoxic effects of lavender and immortelle EOs against different clinical strains of bacteria and fungi were evaluated by Mesic et al. (2018). LEOs were found to demonstrate significant growth inhibition against all tested Gram-positive and Gram-negative bacteria, including MDR strains of microbes, extended-spectrum  $\beta$ -lactamase (ESBL) producing *E. coli* and MRSA [78].

LEO also has potent antifungal properties against a wide spectrum of different yeasts and dermatophytes [76,78]. Multiple studies have suggested that the antimycotic properties exhibited by LEOs are the results of inhibition of biosynthesis of ergosterol, which is one of the vital components of plasma membrane in most of the fungi. This leads to destruction of the fungi cell membrane and eventually, apoptosis ensues [79,80]. D'Auria et al. (2005) reported the use of LEOs against different clinical strains of *Candida albicans* demonstrated both fungistatic and fungicidal activity in a concentration-dependent manner [81]. *C. albicans*, which is a common opportunistic pathogen found in patients who are usually immunocompromised, is said to exhibit its virulence and pathogenicity via the constant reversible transition between the hyphal and yeast form. This transition process is mediated by the formation of germ tube and the application of LEOs was said to be able to suppress the germ tube formation, hence slowing down the spread and progression of the fungal infection [81,82].

Certain degree of antiparasitic benefits in LEOs were observed in a few studies, where Moon et al. (2006) conducted a study to evaluate the antiprotozoal activities of LEOs against *Trichomonas vaginalis*, the primary cause of non-viral sexually transmitted illnesses and *Giardia duodenalis* or *Giardia lamblia*, an important cause of acute and chronic diarrhoea found usually in contaminated food or water [83–85]. LEOs have been shown to inhibit the growth of both *G. lamblia* and *T. vaginalis* in vitro completely, even at low concentrations [83]. Furthermore, LEOs were shown to possess antileishmanial properties when the use of LEOs in different concentrations were effective in inhibiting the activity of *Leishmania major* promastigotes and significantly reducing the number of amastigotes found in the macrophages [86]. Antischistosomal benefits were noticed in a study conducted by Mantovani et al. (2013), whereby incubation with LEOs exerts considerable effects against adult *Schistosoma mansoni* worms and exponentially decreases the rate of egg development after 120 h [87].

### 3. Combination Therapy Involving LEO

Many in vitro studies in the past have demonstrated that the combination therapies involving EOs are beneficial in potentiating their antimicrobial properties and has been countlessly recommended as a potential strategy in mitigating the worsening of AMR [88,89]. Combinatory therapy involving LEOs can be classified into three main forms of drug interaction, i.e., additivity, antagonism, and synergism. Additivity or non-interaction is said to occur when two different bioactive compounds used in combination, produces



antimicrobial effects that are equal to the sum of the individual drugs [90]. When there is pronounced decline in the efficacy of the combination therapy as compared to its individual compounds, it is termed as antagonism [91]. Previous studies have hypothesized that an antagonistic interaction occurs in combination therapy involving LEOs due to combination of bacteriostatic and bactericidal agents at the same time, use of two compounds with similar mechanisms of action or presence of unfavourable physiochemical properties [92]. On the other hand, synergism, which is most favourable and preferred approach of all three, is when the combined effects of both antimicrobial agents are greater than the sum of the effects of the two individual compounds [93]. Numerous studies involving the combination therapy with LEOs in the past have given special attention to the presence of synergistic interactions due to the utilization of multitargeted antimicrobial activity, which results in marked reduction in toxicity and higher efficacy of LEOs [94,95]. Antimicrobial effects of LEOs can be augmented by employing a few different combinations: (i) between different constituents of LEOs; (ii) LEOs with other EOs; and (iii) LEOs with other antimicrobial agents.

To establish the presence of synergism between LEOs and other agents as mentioned above, different in vitro methods are used to evaluate the antimicrobial interactions in these combinatory therapies. However, the most commonly used techniques for synergy prediction is the checkerboard assay and time-kill curve methods [62,96]. Checkerboard method involves multiple combinations of LEO and other test agents in serial dilutions into different microtiter plates. The LEO combination in which the growth of microbes tested is completely inhibited will be the effective MIC value [97]. Data from the checkerboard assay expresses the antimicrobial interactions of these two compounds on the basis of plotting of isobolograms or determination of fractional inhibitory concentrations (FIC) and FIC index (FICI) [98]. The value of FIC can be expressed and calculated using the following equation:

$$FIC_{LEO} = \frac{\text{MIC of LEO in combination}}{\text{MIC of LEO when used alone}}$$

The value of FICI is obtained by addition of the FIC values of the LEO and the other compound:

$$FICI = FIC_{LEO} + FIC_{\text{other compound}}^*, \quad (1)$$

where  $FIC_{\text{other compound}} = \frac{\text{MIC}_{\text{other compound in combination}}}{\text{MIC}_{\text{other compound when used alone}}}$ .

\* other compound denotes substances like EOs other than LEO, constituents of LEO or conventional antibiotics.

Generally, synergistic interaction is said to be achieved when the FICI is equal or less than 0.5, additive or no interaction was seen if the FICI value was between 0.5 and 4.0 and antagonism was portrayed when the FICI is more than 4.0 [99].

The time-kill curve method allows determination of bactericidal effects of each individual compound by measuring the number of viable inoculums in the presence of a certain combination of antibacterial agents at multiple intervals [100]. Although it is time-consuming and labour-intensive, time kill assay is usually deemed as the “gold standard” in synergy prediction due to its good reproducibility and sensitivity [95].

### 3.1. LEO and Other Essential Oils

One of the biggest studies conducted with regard to combinatory therapy involving LEOs and other EOs was performed by de Rapper et al. (2013), where they evaluated the antimicrobial activity of LEO in combination with 45 other aroma-therapeutic oils against three different microbes: *S. aureus*, *P. aeruginosa*, and *C. albicans*. Upon investigating different ratios of different EOs in combination, FICI analysis revealed favorable interactions, whereby 26.7% of these interactions are synergistic and 48.9% are additive. Only one combination exhibited antagonistic effects (LEO and *Cymbopogon citratus*) with FICI value of 6.7. It was also found that the most optimal synergistic interactions were noted in combinations of LEO with *Cinnamomum zeylanicum* and LEO with *Citrus sinensis*.

when used against *C. albicans* and *S. aureus* [101]. In another study, Imane et al. (2020) assessed the antimicrobial benefits in a formulation containing the combination of three different EOs, which are LEO, *Artemisia herba alba* and *Rosmarinus officialis* EOs against common wound pathogens. Disc-diffusion assay revealed the combination of these three EOs have bactericidal effects against all the tested microbes. A synergistic effect was also seen in this combination with FICI values ranging from 0.015 to 0.5 [102]. On the other hand, Abboud et al. (2015) conducted a study to ascertain the antimicrobial activities of combined LEO and *Thymus vulgaris* EOs against common *Streptococcus* and *Staphylococcus* strains that cause bovine mastitis. Mixture of LEO and *T. vulgaris* EO has successfully demonstrated a significant decrease in these bacterial colonies in different samples of cow milk [103]. Orchard et al. (2019) conducted an in vitro study to assess the antifungal activity of 128 different combinations of EOs including LEOs against topical fungal pathogens like *C. albicans* and dermatophytes, which commonly cause superficial fungal infection like onychomycosis and ringworms. Broth microdilution methods were utilized and it was found that most of the combinations with LEOs have fungistatic or fungicidal effects against the fungal pathogens. However, from the isobologram studies, most of the interactions resulted in additivity which is slightly different as compared to previous studies [104]. Another similar study done by Cassella et al. (2002) has proven that the combination of tea tree oil (*Melaleuca alternifolia*) and LEO demonstrated significant antifungal activity against tested dermatophytes like *Trichophyton rubrum* and *Trichophyton mentagrophytes*. Isobologram and FICI analysis further revealed that the combination of *M. alternifolia* EO and LEO exhibit a synergistic antimycotic effect against both tested fungal pathogens [105]. Table 3 illustrates the FICI values of the combinatory therapy involving the use of LEOs and other EOs.

**Table 3.** Synergistic effects exhibited by LEO when used in combination with other EOs from different in vitro studies.

Combination of LEO and Other Essential Oils	Pathogens	MIC of LEO When Used in Combination (mg/mL)	MIC of Tested EO When Used in Combination (mg/mL)	FICI Values	Methods Used to Test for Synergism	Presence of Synergism	References
<i>Cinnamomum zeylanicum</i> (cinnamon)	<i>C. albicans</i>	1.00	1.00	0.40	Checkerboard assay Isobologram	+	[101]
	<i>S. aureus</i>	1.00	1.00	0.50		+	
	<i>P. aeruginosa</i>	1.00	1.00	0.53		0	
<i>Citrus sinensis</i> (sweet orange)	<i>C. albicans</i>	1.00	1.00	0.42	Checkerboard assay Isobologram	+	[101]
	<i>S. aureus</i>	1.00	1.00	0.38		+	
	<i>P. aeruginosa</i>	1.00	1.00	0.51		0	
<i>Artemisia herba alba</i> (desert wormwood)	<i>S. aureus</i>	0.02	0.02	0.03	Checkerboard assay	+	[102]
	<i>E. coli</i>	0.02	0.02	0.25		+	
	<i>P. aeruginosa</i>	0.02	0.02	0.50		+	
<i>Rosmarinus officialis</i> (rosemary)	<i>S. aureus</i>	0.02	0.02	0.13	Checkerboard assay	+	[102]
	<i>E. coli</i>	0.02	0.02	0.25		+	
	<i>P. aeruginosa</i>	0.02	0.02	0.48		+	
<i>Allium sativum</i> (garlic)	<i>C. albicans</i>	0.50	0.50	1.25	Isobologram	0	[104]
	<i>T. mentagrophytes</i>	0.13	0.13	0.23		+	
<i>Syzygium aromaticum</i> (clove)	<i>C. albicans</i>	2.00	2.00	1.50	Isobologram	0	[104]
	<i>T. mentagrophytes</i>	0.50	0.50	4.35		-	
<i>Citrus aurantium</i> (bitter orange)	MRSA	1.00	1.00	0.50	Checkerboard assay Isobologram	+	[106]
	<i>E. coli</i>	2.00	2.00	1.00		0	
	<i>P. aeruginosa</i>	0.75	0.75	0.75		0	

+ indicates synergy; 0 indicates additivity; - indicates antagonism; MRSA: methicillin-resistant *Staphylococcus aureus*.

### 3.2. LEO and Antimicrobial Agents

Several studies in the past have demonstrated mostly additive or synergistic activity in combination therapy involving LEOs and conventional antibiotics. The incorporation of natural products (including LEOs) into different antibacterial agents in treating MDR-pathogens has been shown to cause irreversible disruption of bacterial cell membrane [107,108]. These hydrophobic compounds have the propensity to neutralize the lipopolysaccharide (LPS), which is found in the outer membrane of most Gram-negative bacilli. This will subsequently potentiate the bactericidal effects of the combined an-

timicrobial agent by promoting the influx of these agents into the bacterial cell [109,110]. Yap et al. (2014) reported synergistic interactions between LEOs and piperacillin against MDR-resistant *E. coli* J53 R1 where time-kill analysis revealed complete eradication of the bacteria. The results also indicated the LEO-piperacillin may have a role in reversing the *E. coli* resistance to piperacillin via its anti-quorum sensing effects and ability to alter *E. coli*'s outer membrane permeability [111]. A similar study was conducted involving transcriptomic analysis on the similar strains of MDR-*E. coli* to identify any presence of transcriptional changes to the MDR-*E. coli* genome upon the use of combination of LEO-piperacillin treatment [112]. Pathway enrichment analyses revealed that LEO-piperacillin use causes upregulation of certain genes which affects the biosynthesis of LPS of the bacterial cell wall and the metabolism of *E. coli* in diverse environments, which increases its susceptibility to cellular destruction [112]. In another study conducted by Kwiatkowski et al. (2020), LEO combinations with octenidine dihydrochloride (OCT), an antiseptic agent with broad bactericidal effects were thoroughly investigated. The efficiency of this combination against *S. aureus* ATCC 43300 (reference strains) and other clinical isolates was assessed with checkerboard assays and time-kill curve methods; the FICI was found to be between 0.11 and 0.26, indicating a strong synergistic effect. Further Fourier transform infrared (FTIR) spectroscopy revealed that the combination of LEO/OCT causes modification of cell wall in MRSA, augmenting the penetration of LEO/OCT into the cells [113]. On the other hand, LEO along with the use of chloramphenicol exhibited clear synergism against the Gram-negative *P. aeruginosa*, with the FICI of 0.29. Isobologram analysis further revealed that LEO was able to interact synergistically with many of the conventional antibiotics when combined in ratios with higher proportions of LEO. This is probably the first huge-scaled study focusing on the beneficial effects of LEO when used in combination with other antimicrobial agents [114]. Another study conducted by Yang et al. (2020) detected the presence of synergistic antimicrobial effects when LEOs are used concurrently with meropenem against carbapenemase-producing *K. pneumoniae*, where MIC values of both LEO and meropenem were found to be remarkably decreased. Checkerboard and time-kill assays revealed the FICI to be 0.31 and further proteomic analysis revealed the combination of LEO and meropenem causes disruption of the cellular membrane of *K. pneumoniae* via induction of oxidative stress, resulting in influx of LEO-meropenem and other generated free radicals into the bacterial cell [115]. Other than that, the incorporation of gentamicin into LEO exhibits markedly synergistic interactions when used against different strains of *S. aureus*. In contrast, no interaction was seen when LEO-gentamicin was used against *P. aeruginosa*, which coincides with findings from past studies [116]. Table 4 illustrates the FICI values of the combinatory therapy involving the use of LEOs and other conventional antimicrobial agents.

**Table 4.** Synergistic effects exhibited by LEO when used in combination with other antibiotics from different in vitro studies.

Combination of LEO and Different Antibiotics	Pathogens	MIC of LEO When Used in Combination (mg/mL)	MIC of Antibiotics When Used in Combination (µg/mL)	FICI Values	Methods Used to Test for Synergism	Presence of Synergism	References
Octenidine dihydrochloride	MRSA	0.12	1.71	0.16	Checkerboard assay Time-kill curve	+	[113]
Chloramphenicol	<i>C. albicans</i>	3.00	0.63	1.00	Checkerboard assay Isobologram	0	[114]
	<i>S. aureus</i>	2.00	0.31	0.75		0	
	<i>P. aeruginosa</i>	2.00	0.31	0.29		+	
Ciprofloxacin	<i>S. aureus</i>	2.00	0.11	0.49	Checkerboard assay Isobologram	+	[114]
	<i>P. aeruginosa</i>	2.00	0.04	0.74		0	
Meropenem	Carbapenemase-resistant <i>K. pneumoniae</i>	6.30	8.00	0.31	Checkerboard assay Time-kill curve	+	[115]
Gentamicin	MRSA	0.13	0.13	0.14	Checkerboard assay	+	[116]
	<i>S. aureus</i>	0.64	0.13	0.19		+	
	<i>P. aeruginosa</i>	2.00	0.50	0.70		0	
Piperacillin	<i>E. coli</i>	1.30	0.13	0.26	Checkerboard assay	+	[117]
Ceftazidime	<i>E. coli</i>	5.00	0.50	1.00	Checkerboard assay	0	[117]
Ketoconazole	<i>C. albicans</i>	0.16	0.06	0.53	Checkerboard assay	0	[118]

+ indicates synergy; 0 indicates additivity; MRSA: methicillin-resistant *Staphylococcus aureus*.



#### 4. Significance of Nanotechnology in LEO Use

Nanotechnology refers to the emerging field of molecular studies, dealing with the design, production, and application of materials with size ranged between 1 and 100 nm. Previous publications have shown that the incorporation of nanoparticles into bioactive compounds like LEOs is an effective and feasible strategy in enhancing its antimicrobial effects as these materials may facilitate the delivery of LEOs into the cell, resulting in higher intracellular uptake of LEOs [119,120]. Moreover, the use of nanoencapsulation confers the ability to overcome some of the intrinsic drawbacks of LEOs mentioned previously in this review (i.e., poor oral bioavailability, highly hydrophobic and chemically unstable when being exposed to heat, moisture, or oxygen), allowing the utilization as a potential antimicrobial agent to be fully exploited [36,121]. Hence, over the last decade, nano-based approaches are frequently applied in conjunction with the use of EOs in different disciplines, including in food processing and pharmaceutical industries. The use of nanoencapsulation involving EOs encompasses a wide variety of different nanocarriers designs and materials; however, polymeric nanoparticles (i.e., chitosan and sodium alginate), lipid-based nanoparticles (i.e., liposomes, solid lipid nanoparticles, and micro- and nanoemulsions), and formation of inclusion complexes are some of the common nanosystem platforms employed for the encapsulation of EOs [122,123]. To the best of our knowledge, only a limited range of antimicrobial nanodelivery systems have been utilized into studies involving LEO-based combinatorial therapies. In fact, there is scarcity of in vitro and clinical studies investigating the antimicrobial properties of these LEOs-based nanoparticles against clinically relevant MDR-bacteria, such as *K. pneumoniae*, *P. aeruginosa*, MRSA, and *E. coli* and none reported against strains like *E. faecium* or *A. baumannii*.

One of the common nanoencapsulation strategies used in delivering LEO into the target sites effectively and augmenting its antimicrobial effects is via the formation of molecular complexes like cyclodextrins (CDs) and their derivatives. CDs are macrocyclic oligosaccharide compounds with a central hydrophobic core and outer hydrophilic surface, which plays a role in increasing the chemical stability of LEO [124]. Previous studies with other EOs have shown that complexation with cyclodextrins allows a more sustained and controlled release of the EOs and may potentiate their antimicrobial effects [125,126]. Yuan et al. (2019) investigated the biochemical properties and antimicrobial capacity of LEO when encapsulated in hydroxypropyl- $\beta$ -cyclodextrin (HPCD) in comparison with non-encapsulated LEO against strains of *E. coli*, *S. aureus*, and *C. albicans*. Disc diffusion assay revealed both high bactericidal and fungicidal effects exhibited by the combination of LEO and HPCD composite against all three tested pathogens. The MIC levels of LEO/HPCD composite against those tested microbes were considerably lower when compared to the values obtained when using LEO or the composite extract alone. This marked growth in biocidal activity may be attributed to the increased LEO aqueous solubility after HPCD encapsulation, which facilitates the access of LEO into the bacterial cytoplasm and cell membrane [127]. Similar study was done by Das et al. (2019) whereby four different essential oils including LEO were encapsulated with randomly methylated  $\beta$ -cyclodextrin (RAMEB). The findings from this in vitro study are in accordance with the results obtained from Yuan et al., as the LEO-RAMEB inclusion complexes demonstrated remarkable antibacterial properties against *E. coli* and *S. aureus*; the antimicrobial activities were found to be elevated by at least two to four folds as compared with LEOs only [128].

Over the past decade, there has been a steady increase in publications focusing on the use of various types of polymeric nanofibres as a medium for the delivery of EOs because they demonstrated promising wound healing and antibacterial benefits [129,130]. The fabrication of these nanofibres via the electrospinning technology is considered the most versatile and feasible process where this technique is frequently adopted by fellow scientists [131]. Balasubramanian and Kodam (2014) incorporated LEOs into electrospun polyacrylonitrile (PAN) nanofibrous mats where the process of electrospinning was further facilitated by the addition of an electrolytic solution of sodium chloride with various concentrations, ranging from 0.1% to 0.3%. The antibacterial efficacy of these LEO/PAN

nanofibres against different strains of *S. aureus* and *K. pneumoniae* were evaluated via disc diffusion assays and unsurprisingly, the combination of LEO/PAN exhibited clear zones of inhibition against both bacteria with MIC value of 0.1 mg/mL, which signifies excellent antibacterial activity. Cytotoxicity tests via MTT assay also revealed that the use of LEO/PAN nanofibres results in 100% cellular viability, even at a high concentration of 200 µg/mL, which may suggest that PAN are suitable nanocarriers for medical applications with a low risk for cellular damage [132]. On the other hand, the biosynthesis of silver nanoparticles (AgNP) as an alternative disinfectant and antimicrobial agent has been widely described in many studies, due to its potent bactericidal properties against both Gram-positive and Gram-negative pathogens [133,134]. Sofi et al. (2019) engineered nanofibre-based wound dressings where AgNP and LEOs are simultaneously incorporated into polyurethane nanofibres. These nanofibrous wound dressings fabricated with LEOs and AgNP exhibited significant bactericidal activity against different isolates of both *S. aureus* and *E. coli*. From the in vitro tests as well, gradual increase in concentrations of both LEOs and AgNP demonstrated larger zones of inhibition for both microbes, which may be attributed to the presence of synergistic effects when these two components are combined together. From studies done on other nanoparticles, the addition of AgNP to LEOs is also said to be able to overcome the problems encountered when using polymers such as PAN and sodium alginate nanofibres, such as the presence of narrow spectrum antibacterial properties or low tensile strength [135]. Therefore, these LEOs-AgNP-polyurethane nanofibres wound dressings have a great potential in promoting wound healing and possessing remarkable bactericidal effects against commonly seen skin pathogens.

Other forms of nanoformulations like rhamnolipid-based emulsions and inclusion of hydroxyapatite nanoparticles are becoming increasingly popular when used in combination with LEOs as these nanocarrier systems have been indicated to enhance the antimicrobial potentials of LEOs [136,137]. For the purpose of this review, studies pertaining to the antimicrobial benefits of LEOs when incorporated into different types of nanoparticles against clinically relevant pathogens are summarized in Table 5.

**Table 5.** The use of LEOs in combination with different nanocarrier systems in boosting their antimicrobial activities.

Encapsulation Method	Encapsulating Agent	Target Pathogens	Antimicrobial Activity	References
Inclusion complexes formation	Cyclodextrin (HPCD, RAMEB)	<i>S. aureus</i> <i>E. coli</i> <i>C. albicans</i>	Increases LEOs aqueous solubility, which promotes penetration into cells.	[127,128]
Nanofibres electrospinning	Polyacrylonitrile (PAN)	<i>S. aureus</i> <i>K. pneumoniae</i>	Causes membrane disruption. Inhibition of bacterial growth.	[132]
	AgNP + polyurethane	<i>E. coli</i> <i>S. aureus</i>	Causes membrane disruption. Inhibition of bacterial growth. Exhibits synergistic antimicrobial effects.	[135]
Nanoemulsion	Rhamnolipids	MRSA <i>C. albicans</i>	Increases LEOs aqueous solubility, which promotes penetration into cells. Causes membrane disruption.	[136]
	Refined, bleached and deodorized sunflower oil (RBDSFo)	<i>S. aureus</i> <i>B. subtilis</i> <i>E. coli</i> <i>S. enterica</i>	Causes membrane disruption. Inhibition of bacterial growth. Exhibits synergistic antimicrobial effects.	[138]
Nanoencapsulation	Hydroxyapatite	<i>E. coli</i> ESBL <i>E. coli</i> ATCC 25922 <i>S. aureus</i> MRSA	Causes depolarization of bacterial cell membrane. Inhibition of bacterial growth.	[137,139]
Nanoprecipitation	Starch nanoparticles	<i>E. coli</i> <i>S. aureus</i>	Causes membrane disruption. Inhibition of bacterial growth.	[140]

## 5. Current Challenges and Future Prospects

It is without a doubt that different strategies or approaches have to be adopted in order to slow down or mitigate the acceleration of AMR. One of the main challenges in coming up with an effective solution is that there is no “one-size-fits-all” approach in circumventing this issue. A multitude of strategies and therapies have to be applied concurrently in order to have the maximum therapeutic benefits due to the presence of a multifaceted antimicrobial mechanism. LEO and its derivatives have been shown to exhibit a broad spectrum of antimicrobial activities against many different Gram-positive and Gram-negative bacteria, fungal pathogens, and parasites. Studies in the past have also demonstrated the therapeutic benefits of combinatorial therapies involving LEO. However, there is a scarcity of information regarding the pharmacokinetics and pharmacodynamics of these combinatorial therapies involving LEO. More clinical and in vitro studies should be done to reinforce the presence of promising synergistic interactions between LEO and other essential oils or antimicrobial agents. Different clinical trials, including cytotoxicity studies on these combinatory therapies should be conducted to explore the safety and efficacy of these LEO-based formulations, with hope that these can lead to the development of formulations which will be a safe and prospective alternative for the common antibiotics when used in the clinical practice in treating infections caused by MDR-pathogens. Formulation enhancement will also enable the revival of previously sidelined antibiotics due to growing resistance.

Furthermore, there is a heightened interest in developing novel strategies involving nano-encapsulation of LEOs as these nanomolecules are able to compensate for the sub-optimal physicochemical characteristics of using LEOs only. By increasing its chemical stability and solubility in water, encapsulated LEOs have more reliable and potent antimicrobial effects as compared to non-encapsulated ones due to a more sustained and controlled release of these bioactive compounds into the bacterial cells. However, there is paucity in knowledge about the detailed mechanism on how these nanoparticles have the capacity to potentiate the bactericidal and fungicidal effects of these LEOs. Moreover, only a limited array of nanodelivery systems have been explored in combination with the use of LEOs. Hence, future studies should explore the specific mechanisms of action of these nanomolecules in augmenting the antimicrobial potentials of LEOs and possibly, demonstrating any presence of synergism or additivity when LEOs are incorporated into them. More emphasis should also be placed into employing a broader range of different nanocarriers when using LEOs as a therapeutic approach in combatting AMR.

## 6. Conclusions

In summary, the present review has managed to highlight the importance of LEO and its derivatives as novel antimicrobial agents due to its efficacious bactericidal effects against many drug-resistant pathogens, which are the predominant causes of life-threatening hospital-acquired infections. A range of different combinatory therapies involving LEOs which are proven to exhibit potent antimicrobial benefits have been outlined, where some of these formulations may even have the potential to reverse the resistance to common antibiotics in certain bacteria. In addition, this review discussed the different forms of nanodelivery system that are employed in previous research involving LEO, where these nanocarriers have the capacity to potentiate the therapeutic benefits of LEOs. The integration of these diverse approaches may provide knowledge areas which are imperative in mitigating the threats of AMR.

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