

Inhibitory effects of novel formula of oil in water Nanoemulsions on some pathogenic bacteria associated with wound infections

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Abstract: *Staphylococcus aureus* (*S. aureus*), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) have emerged as a serious problematic pathogen that caused wound infections. Antimicrobial nanoemulsions (NEs) are emulsified mixtures of detergent, oil, and water which have broad antimicrobial activity against bacteria, enveloped viruses, and fungi. So, the aim of the current study is to formulate a novel oil in water nanoemulsion and to assess *in vitro* NEs antibacterial activities on common bacteria found in burn and wound infection. Thus, four NEs essential oils (eucalyptus, clove, black seeds and ginger) either alone or as a mixture of two NEs oils were prepared and their antimicrobial activities were investigated, using agar well Diffusion method. The four tested bacterial strains were *S. aureus*, MRSA, *P. aeruginosa*, and *E. coli*. The activity of NEs was higher against MRSA, *S. aureus*, and *P. aeruginosa* than against *E. coli*. The highest level of synergistic and enhancing effect against MRSA was detected using Eucalyptus- Clove NE or Eucalyptus -Ginger NE. In conclusion, this study confirmed that many NEs oils either alone or a mixture of two NEs oils possess *in vitro* antibacterial activity.

Key words: Nanoemulsion oils, antimicrobial activity, bacterial strains, essential oil.

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I. Introduction

Bacteria are the oldest and the most prevalent organisms on earth. They are varied, versatile, and are commensal to all mammals. They can be both crucial and detrimental to health, depending on host interactions. Climate, habitat, ethnicity, genetics, diet, and activity cause the microbiome to fluctuate in diversity and may alter host susceptibility to opportunistic pathogens [1]. *Staphylococcus aureus* (*S. aureus*), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) are opportunistic pathogens that cause severe and life-threatening infections in immunocompromised patients. The Gram-positive bacterium *S. aureus* is mainly responsible for post-operative wound infection, toxic shock syndrome, and food poisoning. MRSA have since 1961 become increasingly prevalent as pathogenic and invasive organism. The Gram-negative bacterium *E. coli* is present in human intestine and causes lower urinary tract infection, coleocystis or septicemia. Several studies have documented increasing resistance rates in *S. aureus* and *E. coli* to antibiotics.

Pseudomonas aeruginosa is one of the most common causes of wound and systemic infections resulting in significant morbidity and mortality in burns patients. These highly resistant strains are often found in burn units [2]. There is a continuous need to discover new antimicrobial compounds with novel mechanisms of action for new infectious diseases. Therefore, researchers are turning their attention to antimicrobial of plant origin.

The use of natural products with therapeutic properties for a long time was the main sources of important therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicines. They contain a wide range of substances that can be used to treat chronic diseases, and infectious diseases [3]. For application studies, essential oil-based emulsion formation is important due to the hydrophobic nature of the oil system, and it is necessary to formulate a soluble nature using an emulsification technique.

Emulsions are mixtures of two immiscible liquids that are stabilized by a surfactant. Emulsion droplets that are extremely small in size, ranging from 50–500 nm, are called nanoemulsion [4]. Nanoemulsion(NE) preparations from essential oils which contained a wide range of substances played a significant role to treat chronic and infectious diseases by inhibiting microbial growth [5]. Furthermore, they have a therapeutic effect on healing the burn and wounds injuries[6].

Antimicrobial NEs are emulsified mixtures of detergent, oil, and water which have been shown to have broad antimicrobial activity against bacteria, enveloped viruses, and fungi at concentrations that are nontoxic in animals[7].When NEs function by fusing with lipid bilayers of cell membranes, the energy stored in the oil-and

detergent emulsion is released and destabilizes the lipid membrane of the bacteria; hence their antimicrobial activity[8].

The antimicrobial activities of oil-in-water nanoemulsions were shown to possess a broad spectrum of activity against bacterial pathogens. So, the aim of the current study is to formulate novel oil in water nanoemulsions and to assess *in vitro* antibacterial activities of the prepared nanoemulsions on common bacteria found in burn and wound infection.

II. Material and method

2.1 Source of materials

Eucalyptus globulus (eucalyptus), *Eugenia caryophyllata* (clove), and *Zingiber officinale* (ginger) and *Nigella sativa* (black seed) were purchased from Hemani Group of companies. 96 well-Microplates, Muller Hinton Agar (MHA), Muller Hinton Broth, phenol red powder, and Ethanol were purchased from Al-Rowad modern establishment for the supply of medical equipment (Jeddah, SA). Polyoxyethylene-20-sorbitan monooleate (Tween 80) and Sorbitan laurate (span 20) were purchased from Al Shafei Medical and Scientific Equipment, Est (Jeddah, SA).

2.2 Bacterial strains

The NEs essential oils were tested against four bacterial strains. They were standard cultures that were obtained from the Culture Collection of Microbiology Lab., King Fahad General Hospital (Jeddah, SA). The tested bacteria were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), Methicillin-resistant *Staphylococcus aureus* (ATCC 52923), *Pseudomonas aeruginosa* (ATCC27853).

2.3 Preculture preparation of bacteria

Bacterial suspensions were prepared with a concentration of 0.5 McFarland Standard. About 3-4 pure colonies of each treated organism (bacteria) were taken with a sterile cotton swab to a glass tube containing 3 ml of sodium chloride (NaCl). After shaking, the sample suspension was compared to a Standard. Accordingly, the concentration was adjusted using either more sterile sodium chloride or bacterial cells until the suspension matched with standard, equivalent to 1.5×10^8 CFU/ml for bacteria [9, 10]

2.4 Preparation of oil -in- water Nanoemulsion

The NE was prepared by mixing 32% (v/v) of surfactant mixtures of span 20 (S 20) and Tween 80 (T 80) at a ratio of 1:1.5, respectively, and 36% (v/ v) of distilled water (D.W). In brief, a suitable fraction of oil and D.W was mixed and heated, followed by the addition of a warm T 80 drop wise along with vortex. The resulting mixture is heated at high temperature up to 70°C with continuous mixing until one phase emulsion (EM) is produced. After that, the warm S20 was added dropwise to the previous mixture with continuous mixing. This solution is mixed and heated continuously until a clear and transparent solution is formed [12].

2.5 Characterization of the prepared NE formulation

The zeta potential (ZP) and z-average diameter of all NE samples were quantified at $25 \pm 0.2^\circ\text{C}$ by Zetasizer (3000 HS, Malvern Instruments, Malvern, UK) [13].

2.6 Agar well Diffusion method

The antimicrobial activity of the NEs was determined using agar well diffusion method [10, 14].. Inoculated Petri dishes (85 mm×15 mm) were prepared by adding 15 ml of sterile Mueller Hinton agar in each dish and after solidification, 0.1 ml of the bacterial inoculum (10^8 CFU/ml) was spread using cotton swap over the surface. Agar wells (diameter of 5 mm) was made using sterile cork poorer. Each well was filled with 50 μ l of the NEs. After 45 min. at room temperature, the plates were incubated at 37°C for 24 h. Inhibition of bacterial growth was measured as inhibition zone diameters (mm) and the average value was calculated. All experiments were carried out in triplicate

III. Statistical analysis

Statistical analysis will conduct using SPSS software (version 22.0. Armonk, NY: IBM Corp). Statistical Analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's test as a post hoc test and independent t-test for comparison between groups. All Values were expressed as mean \pm standard deviation (SD). Differences Were considered significant if $p \leq 0.05$.

IV. Result

Four Nanoemulsions (NEs were prepared from essential oils (eucalyptus, clove, black seeds and ginger, either alone or as a mixture of two oils), water, surfactants, and cosurfactants. They were characterized and their mean diameters were calculated. The mean diameter was ranged from 50- 300 nm. Their antimicrobial activities were investigated, using agar well Diffusion method.

4.1 Antibacterial Sensitivity of the Nanoemulsions

Nanoemulsions currently investigated displayed variable activities towards the tested bacterial strains. Antibacterial activity was determined by measuring the diameter of the transparent area around each well. The antibacterial activity of eucalyptus and the other four essential oils NEs are summarized in Table 1 and Figure1. Diameter of inhibition zone of essential oil of eucalyptus NE varied from (19.83-12.83 mm). The largest zone of inhibition was obtained for MRSA (19.83±0.288 mm) and the lowest for the *E. coli* (12.83 ±0.288 mm). The present study proved that eucalyptusNE had significant increased inhibition on MRSA (p value< 0.005) as compared to the other NEs (clove, black seeds, and ginger). Moreover, eucalyptus NE showed significant inhibition on the *P. aerogenes* (p value< 0.005) as compared to clove, black seed and ginger NEs. Diameter of inhibition zone of essential oil of eucalyptus NE for *P. aerogenes* was 18± 1.0 mm and for *E. coli* was 12.83± 0.288 mm (p value< 0.005) as compared to the NE of clove, black seed, and ginger oil.

On the other hand, clove, coconut, and black seed showed no activity against *E. coli*. While ginger demonstrated medium activity against the same strain, the diameter of inhibition zone was 10.83± 0.288 mm. Ginger oil NE showed significant increase inhibition on the *E. coli* (p value < 0.005) as compared to the NE clove, black seed, and coconut oil. In the current study, ginger NE has the same inhibitory effect on MRSA, *S. aureus*, and *P. aerogenes*(14.83±0.28).

So that, the majority of NEs possessed antimicrobial activity, but in very different ranges. In general, the activity of NEs was higher against MRSA, *S. aureus*, and *P. aerogenes* than against *E. coli*.

4.2 Antibacterial activity of the mixture of nanoemulsion of two oils

Antibacterial activity for a mixture of two NEs was determined using well diffusion agar methods. According to the obtained results, the combination of eucalyptus NE with clove, black seed, and ginger NEs showed synergistic antimicrobial activity against the four tested bacteria (Table 2, Figure 2).

In the current study, clove and black seed NEs did not show antibacterial activity against *E. coli*. But ginger has the highest inhibition zone with eucalyptus against *E. coli* (12.83±1.4) (Figure 2). In addition, ginger NE alone and mix ginger NE showed significant increase diameter of inhibition zone on MRSA (p value < 0.005) compared to *E. coli* (Figure.3). In addition, an increase in inhibition areas was also found using black seed combined with eucalyptus against *P. aerogenes* where the inhibition zone had changed from 16±1 to 20.33±0.5 mm. Moreover, the mix black seed oil NE showed significant increased inhibition zone (p value < 0.005) compared to the clove, ginger, and coconut oil NEs (Figure 4). While mix black seed NE showed significant increase inhibition on *S. aureus* and *P. aerogenes* (p value < 0.005) as compared to the black seed NE alone (Figure5).

Interestingly, that mix clove NEs demonstrated the same inhibition zone area with MRSA and *S. aureus*(17.66±0.5) (Figure 2). Also, mix clove NEs and clove NE alone showed the same inhibition zone on *P. aerogenes*. Moreover, mix clove NEs showed significant increase of inhibition zone (p value < 0.005) on MRSA and *S. aureus* as compared to clove NE alone (Figure 5).

Table 1: The inhibition zone diameter obtained by nanoemulsion- essential oils against different bacteria

Nanoemulsion essential oil	Diameter of inhibition zone in one essential oil (mean ± SD)			
	MRSA	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Eucalyptus	19.83±0.29 b,d	16.67±2.89 a, d	18±1.0 d	12.83 ± 1.2 a, b, c
Clove	15.67±0.58 c, d	14.83±0.29 c, d	13±1.73 a, b, d	0.00 a, b, c
Black seed	0.0 b, c	11.67±0.58 a, c, d	16±1 a, b, d	0.0 b, c
Ginger	14.83±0.29 d	14.83±0.29 d	14.83±0.24 d	10.83±0.29 a, b, c

Data are expressed as mean ±SD (n = 3), values followed by different letters (a, b, c, d) means that there is significant difference by Duncan test (P< 0.05)

Table 2: The inhibition zone diameter obtained by two nanoemulsion- essential oils against different bacteria

Diameter of inhibition zone for Mixed nanoemulsions- essential oils (mean ± SD)				
Nanoemulsion essential oils	MRSA	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
Eucalyptus- Clove NE	17.67±0.58 c, d	17.67±0.5 c, d	13.0±2.0 a, b, d	0.0 a, b, c
Eucalyptus-Black seed NE	11.67±2.89 c, d	14.33±1.1 c, d	20.33±0.58 a, b, d	0.0 a, b, c
Eucalyptus - Ginger NE	16.33±1.53 d	14.33±1.5	14.33±1.7	12.83±1.4 a

Data are expressed as mean ±SD (n = 3), values followed by different letters (a, b, c, d) means that there is significant difference by Duncan test (P < 0.05)

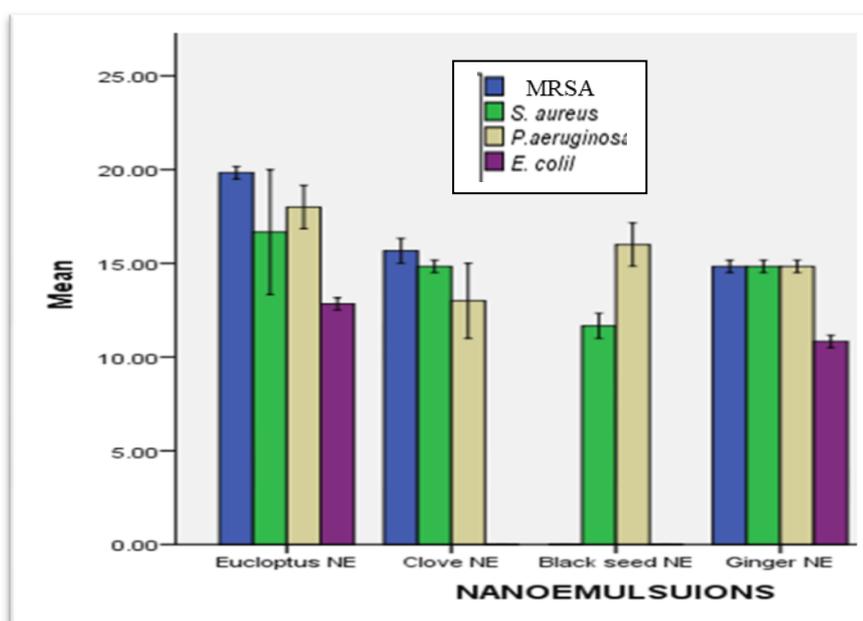


Figure 1. Antimicrobial activity evaluation of the essential oil NEs against MRSA, *S. aureus*, *P. aeruginosa*, and *E. coli* using the agar disc diffusion method.

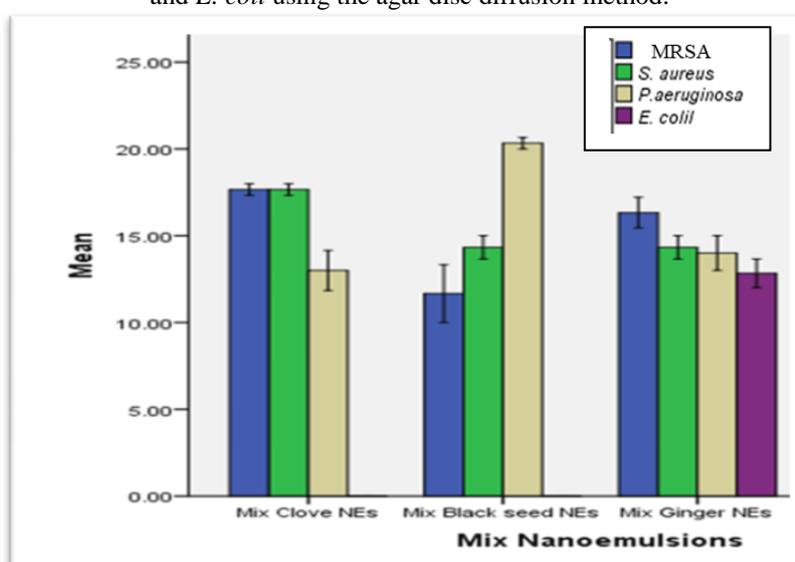


Figure 2. Antimicrobial activity evaluation of the mix two essential oil NEs against MRSA, *S. aureus*, *P. aeruginosa*, and *E. coli* using the agar disc diffusion method.

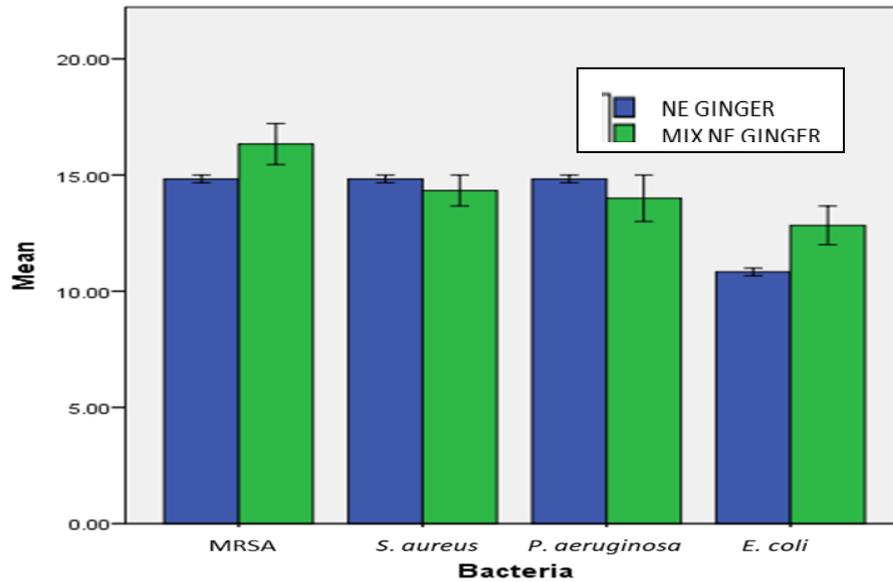


Figure 3: Antimicrobial activity evaluation of the ginger oil NE alone and mixture of ginger + eucalyptus NEs against MRSA, *S. aureus*, *P. aeruginosa*, and *E. coli* using the agar disc diffusion method.

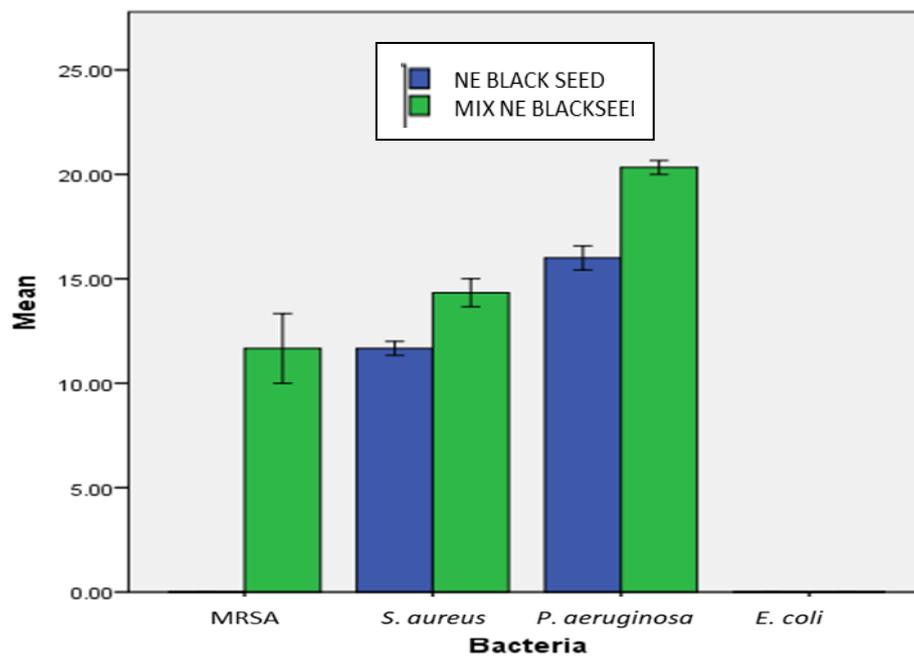


Figure 4. Antimicrobial activity of the black seed oil NE alone and mixture of black seed +eucalyptus NE against MRSA, *S. aureus*, *P. aeruginosa*, and *E. coli* using the agar disc diffusion method.

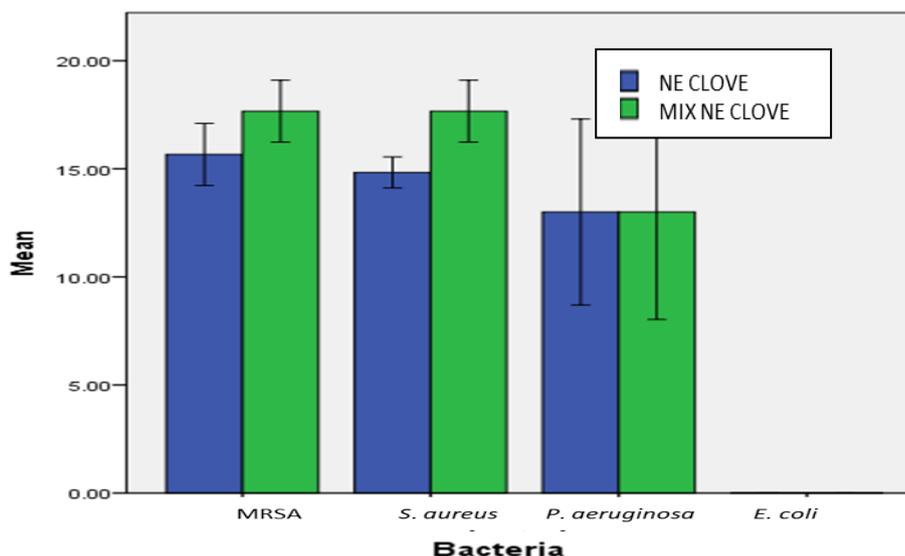


Figure 5. Antimicrobial activity of the clove oil NE alone and mixture of clove+ eucalyptus essential oil NEs against MRSA, *S. aureus*, *P. aeruginosa*, and *E. coli* using the agar disc diffusion method.

V. Discussion

The progress and prevalence of bacterial resistance to antibiotics is a global concern. With the increase of antibiotic resistance, antimicrobials plant products have gained attention in the scientific research. The use of natural antimicrobial compounds is important in the control of human diseases [15].

Medicinal plants are considered an important source of new chemical substances with potential therapeutic effects. They contain a wide range of substances that can be used to treat chronic and infectious diseases [3].

Some studies have already evaluated the antibacterial activity of EOs, and they have shown their potential to fight pathogenic bacteria [16, 17].

Moreover, they mentioned the important of the combination of two essential oils as these combinations may also control some bacteria that are known to show consistently high resistance to plant antimicrobials, such as *Pseudomonas* spp. Also, this study matching Preliminary studies showed that the combination of two EOs had a greater efficacy than the EOs separately [14].

Nanoemulsions have been used in most forms of drug delivery, namely topical, ocular, intravenous, intranasal and oral delivery. These applications leverage the lyphophilic nature of nanoemulsions to solvate water-insoluble drugs; and tunable charge and rheology of nanoemulsions to formulate aqueous solutions that can be easily delivered to patient [18]. In the present study, we have evaluated some NEs-essential oils against some pathogenic bacteria that caused wound infections.

The NE has a broad-spectrum activity against bacteria (e.g. *E. coli*, *Salmonella*, *S. aureus*), enveloped viruses (e.g. HIV, Herpes simplex), fungi (e.g. *Candida*, Dermatophytes), and spores (e.g., anthrax).

In the current study, NE was prepared by mixing of surfactant mixtures of span 20 and Tween 80. This result explained by Pathania et al [19]. who reported that oil-in-water emulsions were set up with mixes containing one hydrophilic surfactant Tween 80 and one lipophilic surfactant Span 80. The blend of Tween and Span 80 demonstrated the best synergistic result in resolving nanoemulsions.

Moreover, Rao and McClements [20] stated that the control of lemon oil NEs was enhanced by blending Tween 80 with sucrose monopalmitate. Stable NEs shaped utilizing surfactant which can more often than not be acquired by changing the surfactant composes and their proportions depend upon its blending properties.

In the present study, the majority of NEs possessed antimicrobial activity, but in very different ranges. In general, the activity of NEs was higher against MRSA, *S. aureus*, and *P. aeroengines* than against *E. coli*. These results explained by Zhang et al. [17] and Bhargava et al., [21] who mentioned that the essential oil NE had 10 times more antibacterial activity than the pure essential oil, which suggested that converting the essential oil to Nano-scale particles greatly improved its bactericidal activity. These results also agreed with other recent studies that showed that conversion of flavor or essential oils into nanoemulsions greatly improved their antibacterial activity, e.g. D-limonene and oregano oil. Moreover, Moghimi et al. [22] performed experiments using a model gram-negative bacterium (*E. coli*) to gain a better understanding of the antibacterial activity mechanisms of the essential oil. They reported that the measured MICs of the pure and emulsified essential oils

showed different inhibitory effects against *E. coli*. The nanoemulsion exhibited high inhibitory effects with MICs of 0.4 mg/ml.

Saranya [23] investigated the formulation and characterization of oil in water NE and its potential antibacterial activity against *P. mirabilis*. The NE was formulated using eucalyptus oil, Tween 20 and ethanol by high energy method, Ultrasonication and the mean droplet size was found to be 20.17 nm as confirmed by dynamic light scattering. They found the growth inhibition was found to be 100% when treated with NE as confirmed by dilution plate count and antibacterial susceptibility method.

VI. Conclusion

This study confirms that many NEs oils either alone or a mixture of two NEs oils possess in vitro antibacterial activity against different bacterial pathogens.

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