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In Vitro Inhibitory Effects of Different Formula of Essential Oils against Bacterial Pathogens Associated withBurn and Wound Infections

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Abstract:*Essential oils (EOs) from plants represent an alternative approach in combating antibiotic-resistant bacteria.The objective of this study was to investigate in vitro antibacterial activity of fiveessential oils, Eucalyptus globules (eucalyptus), Eugenia caryophyllata(clove), Nigella sativa(Black seed), Zingiberofficinale(ginger),and Cocos nucifera(coconut) individually and in combination with Eucalyptus globules oil against four pathogenic bacteria that invade burns and wounds. Two Gram-positive bacteriaStaphylococcus aureus (S. aureus) and Methicillin-resistant Staphylococcus aureus(MRSA) and two Gram-negative bacteria Escherichia coli (E. coli) and Pseudomonas aeruginosa (P. aeruginosa) were used as test organisms. The antimicrobial activity was determined usingagar well diffusion method. Eucalyptus and Eugenia oils showed the greatest antimicrobial activities against all tested pathogenic bacteria. In addition, the combination of Eucalyptus oil with other tested oils except Eugenia oil presented synergistic effect against MRSA and S. aureus. The highest level of synergistic and enhancing effect was detected in Cocos nucifera EO in combination with Eucalyptus globulus. Furthermore, the minimum inhibitory concentration (MIC) was estimated for the best result. In general, Gram-positive bacteria were more sensitive to inhibition by plant essential oils than the Gram-negative bacteria. Minimal inhibitory concentrations of oils were determined using Microdilution method and compared. No toxicity was recorded for all tested oils except for Eugenia oil whereas a moderate toxicity was regarded at 7.5 µg/ml using Artemia salina as test organism.Therefore, Essential oils and their combination can be used against pathogenicresistant bacteria associated with burns and wound infections.*

Keywords: *Eucalyptus globules,Eugenia,essential oils, Staphylococcus aureus, Escherichia coli, MRSA, Pseudomonas aeruginosa, Artemia salina*

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I. Introduction

The improvement and prevalence of the resistance bacteria to many antimicrobial agents becomes a world-wide concern as it is one of the most healththreats and great obstacles in the infection treatment. In recent years, the occurrence of bacterial infectious diseases is increasing, mainly in developing countries. World health organization (WHO) account published in 2014 declared that this serious threat is not a prediction for the future anymore, whereas it is taking place right now all over the world and has the potential to impact all people, of any age, in any country [1]. When bacteria change, antibiotics no longer work in people who require them to cure infections. Numerous illnesses which had been controlled formerly,have appeared again and escaped from human control. The antibacterial activities of essential oils against microorganisms have been described by many researchers [2]. Moreover, the consumption of traditional medicine is highly demanded due to its eco-friendly and cost-effective preparation[3]

There is a high demand by consumers to utilize herbal and microbial origin instead of artificial preservatives in food products as there is always a chance to have foodborne diseases in food factories [4]. Hence, there is a need to develop novel cost-effective treatments or drugs with minimal side effects. The antimicrobial efficacy of many essential oils has been known for many years[5].It has been reported that herbal oils such as,*Eucalyptusglobules* (eucalyptus), *E. caryophyllata*, *Nigella sativa* (Black seed), *Zingiberofficinale* (ginger), and *Cocos nucifera*(coconut)are natural antimicrobial substances that effectively inhibit bacterial

growth [6].These essential oils (EOs) are in great demand in the market [7], since they find applications in burns, cancer, diabetes and wounds [8].

Escherichia coli (E. coli) and *Staphylococcus aureus (S. aureus)* are two opportunistic pathogens that cause severe and life-threatening infections in immunocompromised patients [7]. The Gram-positive bacterium *S. aureus* is mainly responsible for post-operative wound infection, toxic shock syndrome and food poisoning. The Gram-negative bacterium *E. coli* is present in human intestine and causes lower urinary tract infection[9]. Several studies have documented increasing resistance rates in *S. aureus* and *E. coli* to antibiotics [10]. Resistant *Staphylococcus aureus (*MRSA) have since 1961 become increasingly prevalent as pathogenic and invasive organism. These highly resistant strains are often found in burn units [11]. This study is conducting to determine*in vitro* the inhibitory effect of essential oils alone and in combination with *Eucalyptusglobules* oil againstburn and wound bacteria.

II. Materials and Methods

Source of oils

Essential oils of *Eucalyptus globulus* (eucalyptus*), Eugenia caryophyllata*(clove), and*Zingiberofficinale*(ginger) and *Nigella sativa* (Black seed) were purchased form Hemani Group of companies, Karachi, Pakistanand Mountain Rose herbs Company, Eugene, USA.Homemade coconut oil was prepared from the solid meat of coconut.It was crushed, converted to viscous liquid and squeezed through cotton cloth to obtain coconut milk, which was cooled for 48 hours to separate the layers of fat and water. After 48 hours, the fat layer was removed and subjected to mild heating (50°C). The oil obtained through cotton cloth was filtered and used in the present study [13].

Bacterial strains

All bacterial strains evaluated were standard cultures from the American Type Culture Collection (ATCC). *Escherichia coli* (ATCC 25922), *Staphylococcus aureus (*ATCC 29213), MethicillinresistantStaphylococcus *aureus (*ATCC 52923), *Pseudomonas aeruginosa*(ATCC27853) wereobtained from the lab.of Microbiology,king AbdulazizUniversity Hospital,Jeddah,Saudi Arabia. Identification of all isolates were confirmed using some morphological and biochemical characters.

Preculture preparation of bacteria

Bacterial suspensions were prepared at a concentration equivalent to a 0.5 McFarland Standard. About 3-4 pure colonies of each treated organism (bacteria) were taken by sterile cotton swab to glass tube containing 3 ml of sodium chloride. After shaking, sample suspension was compared to a 0.5 McFarland Standard. Accordingly, the concentration was adjusted with either more sodium chloride (NaCl)or cells until the suspension matched that of the 0.5 McFarland Standard, which is corresponding to equivalent to 1.5×10^8 CFU/ml for bacteria [12].

Agar well diffusion method

Agar well diffusion procedure [15] was used to determine the antimicrobial activity of the EOs. Petri Plates (85 mm×15mm) were prepared by pouring 15 ml of sterile Mueller-Hinton agar (MHA) in each plate and the medium was left to solidify. About 0.1 ml of an inoculum suspension (tested bacterium, 10^8CFU/ml) was poured and uniformly spread using cotton swap. After inoculum absorption by agar, well were made using sterile cork poorer (diameter 5 mm) and were filled with 50 µl of the EO prepared inDMSO (v/v). All plates were left for 45 min. at room temperature to allow proper diffusion. The plates were incubated in incubators at 37°C for 24 h. Inhibition of bacterial growth was measured as inhibition zone diameters (mm) and the average value was taken. All experiments were carried out in triplicate.

Determination of minimum inhibitory concentration and toxicity

Minimum inhibitory concentration (MIC) for the five EOs alone (eucalyptus, clove, coconut, ginger and blackseed) and their mixture with eucalyptus were determined using broth microdilution method described by [14] with some modification whereas sterile 96-well plate was used to determine inhibitory activity of each tested oil. Serial dilutions of the concentrated oil wereprepared, and MIC was calculated. Phenol red stain was used as an indicator and was prepared by adding 0.2 mg to 100 ml of deionized water. Bacterial cultures were prepared from overnight culture on MHA and then 2-3 colonies were taken in 3 ml NaCl and the turbidity was adjusted to 0.5 McFarland turbidity standards. The tested sample of EOs was dissolved in DMSO (1:1v/v). Using the microdilution method, 100 µl/well of Muller Hinton broth was added into 12 wells using multichannel pipette. Then, 100 µl/well ofeach essential oil in DMSO or the mixture of two oils was added to well #1 for each

organism and mixed properly. Two-fold dilution of alone EO or mixture of EOs were prepared by transferring 100 µl from well #1 to well #2 and so on and keep diluting and mixing until well #10, and the remain rest 100µl from well #10 was eliminated. Then 25 µl/well of prepared inoculum of bacterial suspension (bacteria 1.5×108 CFU/ml) were added from well # 11 to well # 1. After that 5 µl of phenol red stain was added to all wells from 1 to well 12. Well #11 contained 25 µl of bacteria suspension without EO addition and 100 µl MHB and 5 µl of red phenol stain served as growth control and well #12 contained 100 µl of the medium broth and 5µl of stain (as a control to monitor sterility). Finally, the plates were incubated for 24h at 37°C with shaking. The procedure repeated three times for five EO alone and in combination with eucalyptus oil for all tested bacteria. The MIC was determined as the lowest concentration showing very little growth and was detected by changing the color of the medium as shown in processed plate Fig. 1. Appearance of pink color indicated the MIC.

The MBC (Minimum Bactericidal Concentration) was determined by spreading 100 µl of the cultures on MHA plates and then incubated for 24 h at 37°C. The MBC was determined as the lowest concentration showing no bacterial growth on agar plates. The assays were carried out in triplicate.

The MIC and MBC values were determined by viable counts in MHA, and the MIC was defined as the lowest concentration at which the inoculum viability was reduced up to 90% and MBC was defined as the lowest concentration at which the inoculum viability was reduced up to 99.9% or no apparent growth occurred.Cell toxicity after 8 hrwas determined against *Artemia salina*and surviving percentages (LD50) of larvae was determined [21]

Statistical analysis

Statistical analysis was performed in SPSS (version 22.0. Armonk, NY: IBM Corp). The exhibited MICs and MBCs were grouped according to oil type. All data were the mean of three experiments. Summary of the data was expressed as mean ± standard deviation (SD). Statistical difference among groups were determined using one-way analysis of variance; ANOVA followed by Duncan multiple comparisons test and independent t-test. The significance of the data was determined by the P value. P values greater than 0.05 were considered non-significant, and P values less than 0.05 were considered significant.

III. Results

Antibacterial sensitivity of the essential oils

Essential oils currently investigated displayed variable activities towards the tested bacterial strains. Antibacterial activity was determined by measuring the diameter of the inhibition area (Figure 1).The antibacterial activity of eucalyptusand the other five EOs were summarized in Table 1 and Figure 2. The result proved that eucalyptusEO had significant activity (P value< 0.05) against MRSA and *S. aureus* (Figure 3A)with diameters of inhibition zones of 33±2.6 mm and 24±1.7 mm, respectively. Also, it showed significant antibacterial activity against the Gram-negative *P. aeruginosa*23.3±2.9 mm and *E. coli* 17.8±0.3mm. Rather high activity was also found for clove EO against MRSA (20.3±0.5 mm) and *E. coli* (19.7±0.6 mm). Also, it possessed antimicrobial activity against *S. aureus* and *P. aeruginosa* with diameters of inhibition zones of 17.8±0.3 mm and 13.6±1.2 mm, respectively. Moderate level of activity against MRSA was demonstrated by Black seedEO with diameters of inhibition zones of 12.6±1.2 mm.

MRSA did not show any sensitivity to coconut and ginger oils while Black seed and coconut oils showed moderate activities against *S. aureus* (11.3±1.2 mm) and (10.7±0.5 mm), respectively. Moreover, Black seed and coconut oils showed no inhibitory activity against *E. coli* and *P. aeruginosa*. In addition, ginger EO showed no antimicrobial activity against the four tested bacterial strains while the majority of EOs possessed antimicrobial activity in very wide ranges. In general, the activity of EOs was higher against Gram-positive bacteria, MRSA and *S. aureus* than against Gram-negative bacteria, *P. aeruginosa* and *E. coli.*

Antibacterial sensitivity of the mixture of two essential oils

Antibacterial activity for a mixture of two EOs was determined using well diffusion agar methods. According to the obtained results, the combination of eucalyptus EO with coconut, Black seed, and ginger EOs showed synergistic antimicrobial activity against the four tested bacteria (Table 2, Figure 2). An antagonistic effect was observed in case of using clove oil with eucalyptus against MRSA, *P. aeruginosa*, and *E. coli*.

The highest level of synergistic and enhancing effect against MRSA was detected using coconut witheucalyptus EO (diameter of zone inhibition was 30.7±1.2 mm) (Figure 3B) while coconut oil alone did not show antibacterial activity against MRSA. In addition, an increase in inhibition areas was also found using

coconut oil combined with eucalyptus against *S. aureus* where the inhibition zone had changed from 10.7 to 11.6 mm.

High enhancing effect was recorded using a mixture of eucalyptus and ginger EOs against Gram-positive bacteria. Interestingly, no antibacterial activity was recorded for ginger alone, while in combination with eucalyptus oil, inhibition zone area increased to reach16 mm against MRSA and 16.3 mm against *S. aureus*. It is worth to mention that black seed EO alone did not show antibacterial activity against Gram negative bacteria, but its combination with eucalyptus oil demonstrated noticeable increase in inhibition zone against positive bacteria, MRSA (13.7±1.2 mm) and *S. aureus* (13.7±1.2 mm). Clove EO alone was significantly more active against most strains but in combination with eucalyptus oil demonstrated equal activity against *S. aureus* (17.8±0.3 mm) and showed an antagonistic effect against MRSA where inhibition zone changed from 20.3 to 17.8±0.3 mm. Also, the inhibition zone diameter was decreased against *P. aeruginosa* (12.3±0.6 mm) and *E. coli* (16.7±2.8 mm).

Table 1. The antimicrobial activity of the five EOs against different tested bacteria

Essential oils	**Diameter of inhibition zone alone (Mean ± SD)**			
	S. aureus	**MRSA**	***P. aeruginosa***	***E. coli***
Eucalyptus	24.0^{b}±1.7	33.0^{c}±2.6	23.3^{b}±2.9	17.8^{a}±0.3
Clove	17.8^{b}±0.3	20.3^{c}±0.5	13.6^{a}±1.2	19.7^{c}±0.6
Black seed	11.3^{a}±1.2	12.6^{a}±1.2	ND	ND
Ginger	ND	ND	ND	ND
Coconut	10.7±0.5	ND	ND	ND

Data are expressed as mean +SD (n = 3), ND: No Zone of inhibition,The different letters in each row means that there is a significant difference between each
two types of bacteria at $p<0.05$

Table2. The antimicrobial activity of the mixture of two essential oils against different tested bacterialpathogens

Essential oil	**Diameter of inhibition zone in combination with Eucalyptus oil (Mean ± SD**			
English name	*S. aureus*	MRSA	*P. aeruginosa*	*E. coli*
Eucalyptus+ clove	17.8^{b}± 0.3	17.8^{b}± 0.3	12.3^{a}±0.6	16.7^{b}±2.8
Eucalyptus+ Black seed	13.7±1.2	13.7±1.2	ND	ND
Eucalyptus+ ginger	16.3^{a}±4.0	16.0^{a}±1	ND	ND
Eucalyptus +coconut	11.6^{a}±0.5	30.7^{b}±1.2	ND	ND

Data are expressed as mean ±SD (n = 3), ND: No Zone of inhibition, the different letters in each row means that there is a significant difference between each
two types of bacteria at $p<0.05$.

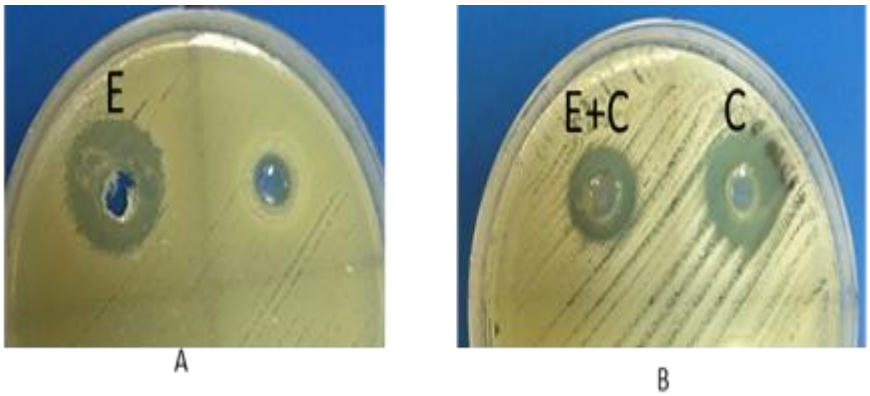

Figure 1: Inhibition zone against *S. aureus*, A: using eucalyptus EO (E), (B): using a mixture of eucalyptus andclove EOs (E+C) and clove oil (C).

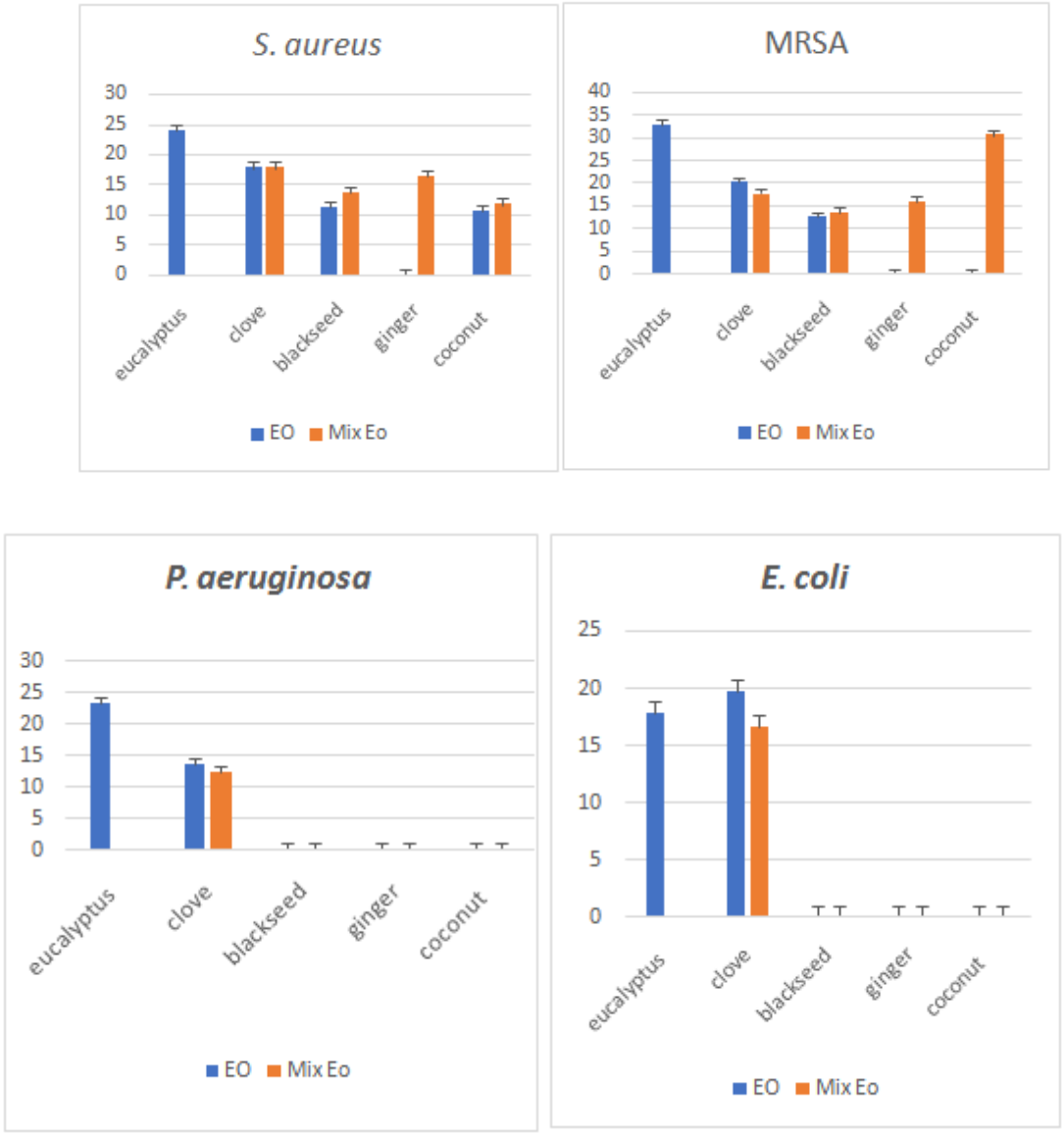

Figure 2: The effect of five essential oils and their mixture with eucalyptus (v/v)against four tested bacteria

Minimum inhibitory concentrations and toxicity of the five essential oils alone

The MICs of the five EOs alone against four tested bacteria were determined using dilution method (Figure 3). The results were summarized in Table 3.Statistically significant differences between the tested essential oils were determined by one-way ANOVA (P value < 0.05). Clove essential oil exhibited the highest significant inhibitory against Gram-positive bacteria(MRSA and*S. aureus*)(P value< 0.05) andexhibited inhibitory effect against Gram-negative bacteria (*P. aeruginosa* and *E. coli)*but not significant (P value >0.05). The MICs for the clove essential oil ranged from 0.2 - 1.1 % (v/v) for all test bacteria. Eucalyptus EO showed MICsand MBCs but with no significant result (P value> 0.05) against MRSA, *S. aureus*, and P. aeruginosa. MIC ranged from 1.5 - 1.8 % (v/v) Similarly, compared to eucalyptus EO activity, the coconut, black seed and ginger EOs showed moderate to low activities against the tested bacteria. For coconut oil, MIC ranged from 5.9- 17.6% (v/v) (P value< 0.05) while MIC of ginger ranged from 7.2 - 17.6% (v/v) (P value< 0.05)but the MIC of black seed ranged from 8.8- 17.6 % (v/v) (P value< 0.05). MBCs values of the essential oils were similar or even higher than the corresponding MIC values.

Minimum inhibitory concentration and toxicity of a mixture of two essential oils

MICs of a mixture of two Essential oils were determined andaccording to the obtained results (Table 4), the combination of eucalyptus EO with coconut, blackseed and ginger EOs showed synergistic antimicrobial activity against the tested bacterial pathogens. The combination of clove with the eucalyptus showed the highest significant antimicrobial activity (P value < 0.05) against the all tested bacteria (MIC ranged from 0.9 – 1.8 %, v/v).The highest level of synergistic and enhancing effect against MRSA was detected using coconut essential oil with *Eucalyptus*(P value< 0.05), the MIC was 1.4 % (v/v) while MICs were5.9 - 17.6% (v/v) for *S. aureus* and *P. aeruginosa*, respectively, which were higher than that of each one alone. Combination of eucalyptus with either ginger or blackseed showed higher MIC compared to the activity of oil alone. For ginger,MICranged from 5.9 -17.6 % (v/v) while MIC of blackseed ranged from 8.8 – 17.6 %,v/v (p value< 0.05).

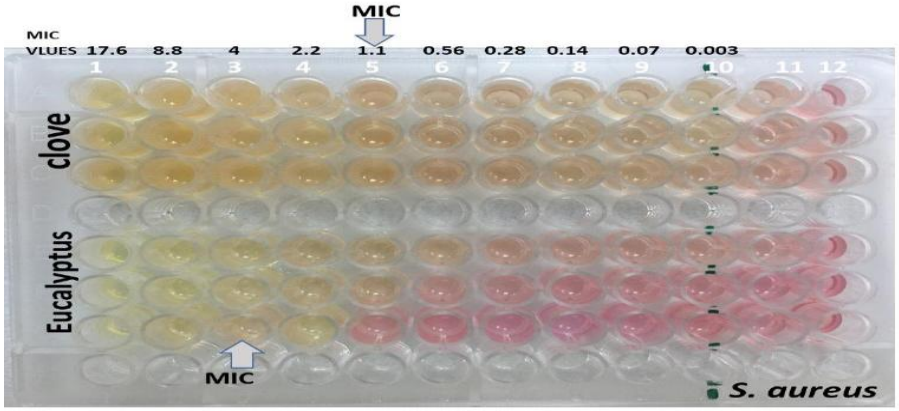

Figure 3: Determination of MIC for EOs (clove & eucalyptus) against *S. aureus*.Appearance of pink color indicated the MIC

The toxicity of different oils or their mixture with eucalyptus was determined after 8 hr. using *Artemia saline* as test organism (Table 5). Toxicity (LD50) was recorded for each tested oil as the concentration that kill 50% of the larva. No toxicity was recorded for blackseed, coconut, gingerand eucalyptus while moderate toxicity was recorded for clove at 7.5 µg/ml. Concerning the mixture of essential oils with eucalyptus, no toxicity was recorded for all tested mixtures except for eucalyptus+ clove whereas moderate toxicity was recorded.

Table 3: The minimum inhibitory and minimum bactericidal concentrations of some essential oils against different tested bacteria

Oil used	**MRSA**		***S. aureus***		***P. aeruginosa***		***E. coli***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Eucalyptus	1.46± 0.63	1.46± 0.6	1.46±0.6	1.40±0 .60	1.83± 0.63	1.83± 0.63	ND	ND
Clove	0.1867c± 0.08	0.186c± 08	1.1acd± 0.0	1.1acd± 0.0	0.74ab± 0.31	0.74ab± 0.31	0.37b± 0.16	0.37b± 0.16
Black seed	11.73d± 5.08	11.73d±5	8.8cd±0. 0	8.8cd±0.0	17.6bd± 0.31	17.6bd± 0.0	ND	ND
Ginger	7.2c± 2.77	7.2±2.77	11.73d± 5.08	11.73d±1. 8	17.6ad± 0.4	17.6ad± 0.0	ND	ND
Coconut	17.6d±0	17.6d±0	5.86acd± 2 .0	5.86acd± 2.2	17.6bd± 0.01	17.6bd± 0.0	ND	ND

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

Table 4: The MICs and MBCs of mixture of two essential oil against different tested bacteria.

Tested oils	**MRSA**		***S. aureus***		***P. aeruginosa***		***E. coli***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Eucalyptus + clove	1.83cd± 0.63	1.83cd± 0.63	1.10d± 0.0	1.1d± 0.0	0.55a± 0.55	0.55a± 0.55	ND	ND
Eucalyptus + Black seed	8.80cd± 0.0	8.80cd±0.0	7.33cd± 0.54	7.33cd± 2.54	17.6abd± 0.0	17.6abd± 0.0	ND	ND
Eucalyptus + ginger	5.86d± 2.54	5.86d± 2.54	5.86acd± 2.5	5.86acd± 2.54	5.86d± 2.54	5.86d± 2.54	17.6abc ±0.0	17.6ab±0.0
Eucalyptus +coconut	1.83bc±0.63	1.83^{b}c± 0.63	5.86acd± 2 .54	5.86acd± 2.54	17.6abd±0 .0	17.6abd ±0.0	ND	ND

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

Table 5: The toxicity (LD50) of the different oils and their mixture with Eucalyptus

Tested oils	LD50 (µg/ml)	Tested oils	LD50 (µg/ml)
Eucalyptus	> 7.5	**Eucalyptus+ clove**	**7.5**
Clove	7.5	**Eucalyptus+ Black seed**	**> 7.5**
Black seed	> 7.5	**Eucalyptus+ginger**	**> 7.5**
Ginger	> 7.5	**Eucalyptus +coconut**	**> 7.5**
Coconut	> 7.5		

IV. Discussion

Universally in developed and developing countries, burns is one of the most damaging conditions in an emergency affecting both genders in all ages [15]. Traditional medicine has a vital role in burn wound repair [16]. In addition, the development and prevalence of resistance to presently available antibiotics is a global concern [17]. With the increase in bacterial resistance to antibiotics, antimicrobials plant products have obtained attention in the scientific research. The implement of natural antimicrobial compounds is important in the control of human diseases and plant microbial origin [18]. Molecules obtained from plants offer an alternative to aid wound healing. Strong evidence about essential oils anti-inflammatory and antimicrobial properties is thoroughly described and characterized in many scientific researches [19].

In this study, the antibacterial activity of various EOs has been studied against various pathogenic bacteria. Combined EOs has obvious advantages such as increasing activity of both agents. In many studies, EO of eucalyptus, clove, Black seed, ginger and coconut demonstrated good antimicrobial properties; however, activity of eucalyptus in combinations with other EOs is not well investigated. In the present study, we investigated activity of these five essential oils (EOs) separately and in combination with eucalyptus essential oil.

Two major representatives' bacterial groups, Gram-positive (MRSA and S. aureus) and Gram-negative (*P. aeruginosa* and *E. coli*) were used as test organisms. Valizadeh et al. (2018) reported that eucalyptus as natural antimicrobial substances can effectively inhibit bacterial growth by morphological destruction of bacteria [20]. The results of this study proved high antimicrobial activity of eucalyptus EO which demonstrated in general higher susceptibility of MRSA and S. aureus. In addition, it showed significant effect against P. aeruginosa and E. coli using well diffusion agar and MICconfirmed that EO of eucalyptus not only possesses antimicrobial activity but also has synergistic antimicrobial activity when combined with other essential oils [5]. In this study, the majority of EOs demonstrated significant differences in activity against the tested bacterial pathogens. The action of EOs alone and their combination with eucalyptus oil showed either synergistic or antagonistic effects.

The plant-based essential oils have been shown to possess antimicrobial, wound-healing, insecticidal, and pharmaceutical properties [21]. According to Enrico et al. (2004) the essential oils, unlike antibiotics, are composed of many molecules so that bacteria cannot resist or has less resistant. Preventively and curatively, they are especially known for their potent antibacterial, antiviral, anti-fungal and anti-inflammatory effects [6]. WHO noted that majority of the world's population depends on traditional medicine for primary healthcare especially the essential oil [22]. An important characteristic of essential oils and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane, disturbing the cell structures and rendering them more permeable to critical molecules and ions which lead to cell death [23].

Three EOs of Black seed, ginger and coconut were significantly more active against MRSA and *S. aureus* than against *P. aeruginosa* and *E. coli*. However, some differences were present as the best synergistic effect was seen in eucalyptus and coconut oils against MRSA. It was found that coconut oil has antibacterial,

antiviral and antifungal activities [24]. Furthermore, coconut oil was able to eliminate bacterial infection and can also stimulate the immune response [13]. Clove oil was significantly inhibited all the tested bacteria in this study. Similarly, the essential oil of Clove is characterized by the most important antibacterial and antioxidant properties due to their phenolic structure [25].

Extensive studies on black seed have been carried out by various researchers and a wide spectrum of its pharmacological actions have been explored which may include antimicrobial, anti-inflammatory and antioxidant properties. Due to its miraculous power of healing Black seed has got the place among the top ranked evidence based herbal medicines [26]. Our results approved that black seed possess antimicrobial activities especially against Gram-positive bacteria MRSA and S. aureus. It was reported that ginger extracts have been extensively studied for a broad range of biological activities including antibacterial, anticonvulsant, analgesic, and other beneficial activities [27]. Many studies have proved that ginger has strong antioxidant antigenotoxic, antimutagenic and anticarcinogenic and was effective when combined with eucalyptus against the growth of Gram-negative bacteria [28].

The minimum inhibitory concentration (MIC), which is a key indicator of an antimicrobial agent's potency, is defined as the concentration (mg /l) at which visible growth of bacteria is prevented under defined growth conditions [29]. The present study showed that the result obtained from MIC was supporting the agar well diffusion method results and approved that all essential oils possessed antimicrobial activity against all the microorganisms tested.

The MICs and MBCs of clove and eucalyptus essential oils in this study were similar to the known literature [30, 31], with a little difference, which could be for several reasons such as a different growing environment of clove and eucalyptus, different extraction methods of essential oils, and so on. Different essential oils have different antimicrobial activity because of their components [32].

The antimicrobial activity of combinations of clove and eucalyptus essential oils has not been reported before. Individual essential oils contained complex components which, when combined with each other, may lead to additive, synergistic or antagonistic effects. The results showed that combinations of clove and eucalyptus essential oils exhibited additive or synergistic effects against all the test strains. The mechanism of antimicrobial activity of mixed essential oils is still not clear and further studies in this area are needed. This result may be useful for the combination of eucalyptus and the other four EOs against special microorganisms in medicine and the food industry.

V. Conclusions

According to the obtained results, the combination of essential oil eucalyptus with different essential oils showed a synergistic effect against MRSA and S. aureus while its mixture with clove oil showed an antagonistic effect. Combinations of EOs provide an effective and economically feasible approach in combating antibiotic-resistant bacteria. However, unlike studies on antibiotic combinations, combinations of EOs are not so widely investigated and future studies should be devoted to the evaluation of EOs combinations against clinical isolates of multidrug-resistant bacteria, and to study the combined effect of different EOs components including also oil components present in small proportions. It is clear that the majority of EOs possessed antimicrobial activity but in very wide ranges. In general, the activity of EOs was higher against positive gram bacteria than against negative gram bacteria.

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