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RESEARCH ARTICLE

Evaluation the Antibacterial Effect of Rosemary and Lemon Grass Essential Oils against Planktonic and Biofilm of MRSA

Zainab Zamel Khalaf*, Ibrahim J. Abed

Department of Biology, College of Science, University of Baghdad, Baghdad-Al-Jadiryia, Iraq.

***Corresponding Author: Zainab Zamel Khalaf**

Abstract

There has been increasing interest in volatile oils in recent years due to the need for new treatments against microbes. Bacterial resistance is widespread worldwide and the first cause of this resistance is the excessive use of antibiotics and bad practices to combat infection in hospitals, making it one of the biggest issues at present. In this study, twenty bacterial isolates of methicillin-resistant *Staphylococcus* bacteria were diagnosed and all were diagnosed using Vitek. The synergistic effect of the rosemary and lemongrass essential oil against these bacteria was studied in the form of suspended or single cells and when they formed the biofilm. Different concentrations of both essential oils were used to experiment the minimal inhibitory (MIC) effect on these bacteria. The effect of each oil was studied separately and then their synergistic effect was investigated. The bacteria's ability to produce the biofilm using the Tissue culture plate method Whether it was strong, moderate, or weak, and then investigated the inhibitory properties of each oil against the biofilm of the bacteria and also studied the synergistic effect of both oil against the biofilm of the bacteria. The findings proposed that all isolates were affected and inhibited with lemon grass essential oil except for one isolate and was an oil effect Lemon grass is larger compared to rosemary essential oil, but the synergistic effect was better in compare with the effect of each oil alone. The results of the biochemistry of the bacteria showed that 15 isolates of *Staphylococcus aureus* were formed of a strong biofilm and 4 isolates formed for a medium biofilm and one isolate only for a weak. The results also find that the lemon grass and rosemary essential oil had an effect against the biofilm but with different concentrations. There were 3 isolates that were not affected by each one of plant oil (3, 6 and 10). The synergistic effect of both essential oils showed that all isolates were inhibited except (6, 9 and 10).

Keywords: *Essential oils; Synergistic effect; MRSA; Antibacterial activity, Biofilm.*

Introduction

When the scientific field was developments, the medicinal characteristics of plants have established a great concern because of their pharmacological activities, low toxicity and economic feasibility [1]. Many studies have focused on the benefits of plant-extracted phytochemicals and their activity on human health. Additives obtained naturally from plants can be compounds, groups of compounds, or essential oils. There has been growing attention in volatile oils in new years due to the require for novel treatments on microbes.

Bacterial resistance is common worldwide and the first reason of this resistance is the excessive use of antibiotics and bad practices to combat infection in hospitals, making it one of the major issues at present. *Staphylococcus aureus* is commensal micro-organism and an important opportunistic human pathogen, causes a variety of hospital and community-associated infections, such as sepsis, pneumonia, endocarditis, bacteremia, osteomyelitis, arthritis and skin diseases [2]. Resistance for antibiotics and biofilm-forming capacity are very important for the development of *S. aureus* as a pathogen in both community settings and healthcare. Biofilms are populations of multilayered bacterial cells, which are growing in an enclosed exo-polysaccharide matrix on a surface.

S. aureus is one of the most common causes of infections regarding implanted medical devices. On the other hand, most of the adhered *S. aureus* are MRSA [3, 4].

MRSA is a serious problem, in the treatment and control as a resultant of multidrug resistant and ability to cause a wide variety of human diseases [5, 6]. Biofilm formation of *Staphylococcus aureus* is the main contributing factor to bacterial antibiotic resistance. *Staphylococcus aureus* becomes resistant by producing an extracellular polymeric substance (EPS) matrix that is composed of polysaccharides, nucleic acids, proteins and lipids [7, 8].

Rosemarinus officinalis essential oil is an important for medicinal treatment and its powerful as antibacterial, cytotoxic, antimutagenic and antioxidant properties. Characteristic of essential oils and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable [9].

Researchers have found that lemongrass holds antidepressant, antioxidant, antiseptic, astringent bactericidal, fungicidal, nervine and sedative properties [10]. Lemongrass oil has antibacterial activity against a diverse range of organisms comprising gram positive and gram negative organism, yeast and fungi [11]. These compounds act over the lipids of the cell membrane modifying its structure and turning it more permeable, allowing the passage of ions and or other substances [12]. Previous studies have shown inhibition of MRSA *S. aureus* and other pathogens by various essential oils, but these oils were not tested synergistically specifically against MRSA.

Thus, the aims of this work were to estimate the antimicrobial activity of *C. citrates* and *R. officianalis* against MRSA *S. aureus* in both planktonic and biofilm forms and the comparison between the effect of each one of these plant oil against planktonic cell and biofilm and see if the synergistic effect between both these oils together gave higher or lower effect against this pathogenic bacteria.

Materials and Methods

Bacterial Isolates and Culture Media

S. aureus isolates were collected from clinical specimens submitted to the clinical diagnostic laboratories, during a 4 -month period, from September to December 2017. Isolates were diagnosed by standard phenotypic tests such as colonial morphology, Gram staining, catalase test, ability to grow on mannitol salt agar, DNase (Merck, Germany) and slide as well as tube coagulase tests [13] to identify as *S. aureus.* Further identification of MRSA was achieved by vitek 2 compact system, isolated bacteria were maintained for long storage on Brain heart medium, by adding 20 % glycerol in -80°C.

Preparation of Essential oil of *C. citratus* and *R. officianalis*

The essential oils of these plants were isolated from air dried leaves (250g) by Cleavenger hydro distillation method. The plant material with D.W (1.2L) was boiled for 3h; the essential oil was kept at 4^0 C until used [14].

In Vitro Inhibitory Activity of Plant Oils on Planktonic MRSA

Overnight ***S. aureus*** cultures contained 1× 10^8 colony-forming units/ml (in accordance to McFarland tube no. (0.5) were grown in Trypticase soya broth (TSB) broth at 37°C for 24 hr under aerobic conditions. These cultures were added to microtiter plate as triplicates for each isolates in a volume 50 µl. After that the serial concentration of plant oils were added to cultures and incubated overnight in 37 C. The results read by nacked eye, the positive control bacteria without oil but the negative control plant oil with broth without bacteria.

Biofilm assay

S. aureus biofilm formation

For study the ability of *S. aureus* for producing biofilm, method described by Maldonado *et al* [15]. Was followed, studied isolates cultured in TSB (Rashmi, India) incubated at 37ºC for 18 hour, after that bacterial culture was added in TSB and used to in compare with MacFarland tube no. 0.5. Two hundred microliters of this bacterial culture were used to inoculate pre-sterilized 96-well polystyrene microtiter plates and later incubated for 24 hours at 37ºC.

After incubation, all wells were washed with distilled water (D.W) for the elimination of unattached cells. Afterward, 200 µl of 1% crystal violet was added to each well, the plates shacked three times in order to facilitate the dye to get the bottom of the well.

Each well was washed with 200 µl D.W after 15 minutes at room temperature, this process was repeated three periods. The binding of crystal violet to biofilm was extracted later with 200µl of ethanol, and then absorbance was detetected at 490 nm in an ELISA reader (Beckman coulter, Austria). Controls represent crystal violet binding to the wells exposed only to the TSB without bacteria. All the assays were performed in triplicates.

Antibiofilm Activity of Plant Oils on MRSA

For the inhibition of biofilm, the biofilm producing isolates of *S. aureus* were selected to be assayed. Same protocol described earlier was followed to produce a biofilm, but the previously prepared plant oils were added before the staining step, these plant oils represent containing media with minimum inhibitory concentration (MIC) to the biofilm containing wells: consequently, the plates was incubated for another 24 hours at 37ºC, after that all wells were washed and stained as the same protocol described above.

Results and Discussion

Bacterial Isolates and Culture Media

A total of 20 isolates were confirmed as *Staphylococcus aureus* (MRSA) (20 human clinical isolates from wounds), were collected and maintained into (TSB) medium then isolates were sub-cultured on to Mannitol Salt Agar (MSA) plates and incubated at 37C° for 24 hr. vitek 2 compact system was also done as confirmation test.

In Vitro Inhibitory Activity of *C. citratus* on planktonic *S. aureus*

The results of the present work proposed that essential oil *of C. citratus* gave antimicrobial effect against *S. aureus* in all its concentrations, the higher effect was observed by higher concentration (10000 µg/ml) that gave no growth for 8 isolates from 20 that observed by naked eye, while lower effect was achieved by lower concentration (2500 µg/ml) which gave no growth for 4 isolates only from 20, while only one isolate not affected by any concentration, as show in figure (1). The antibacterial activity was found progressively increasing with the increase in concentration of oil. The maximum effect was found at concentration 10000 µg/ml and minimum effect was observed at concentration 2500 µg/ml of oil.

The use of methods based on micro dilutions is more intense and seems to be very appropriate to determine the values of MIC and MBC. Essential oils are complex mixtures of a wide diversity of components and their antimicrobial activity is therefore related to their composition, configuration, amount and their possible interaction [16].

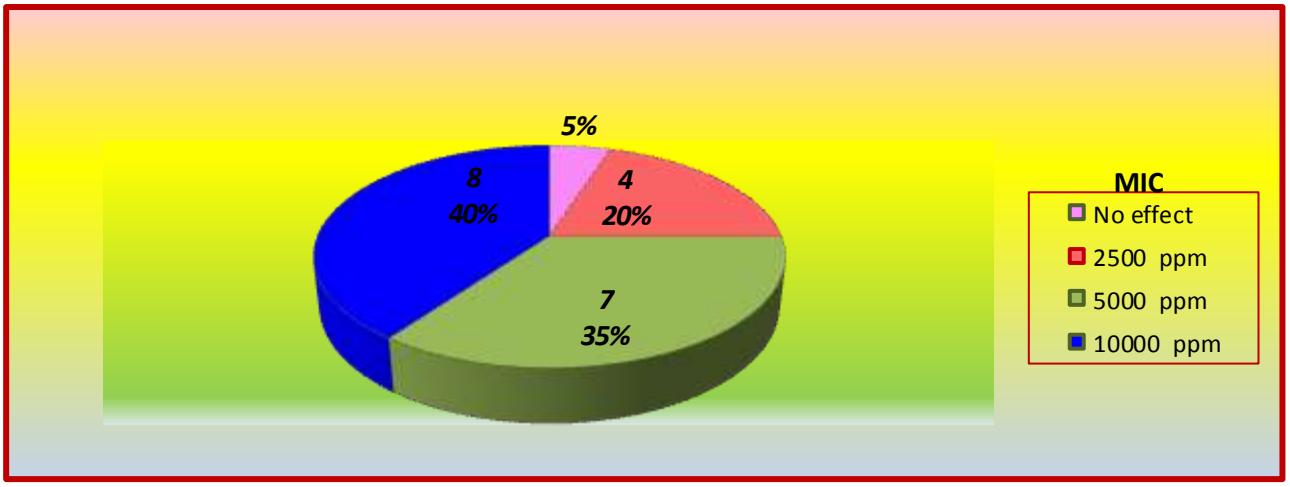

Figure 1: Effect of *C. citratus* essential oil on planiktonic cells

The results of Almeida *et al* [17].Confirmed that the essential oil of *C. citratus* had micro biostatic and microbicidal effect against all tested organisms. The common CFU/ml for the biofilm of *S. aureus*, *S. mutans* and *C. albicans*, whether isolated or in association, was lesser in the group treated with essential oil than in the control one.

These results were in agreement with the results of current study. Antimicrobial effects by using the essential oil of C. citratus, was found by Onawunmi [18], who analyzed the action of essential oil at 0.05% to *S. aureus*, *Bacillus subtilis* and *Escherichia coli* by agar diffusion method. In the work of Hammer *et al* [19].

It was found significant antimicrobial action of oil of *C. citratus* for all organisms analyzed.

In Vitro Inhibitory Activity of *R. officianalis* on planktonic *S. aureus*

The results of the current study reported that essential oil *of R. officinalis* gave antimicrobial effect against *S.aureus* in all its concentrations, the higher effect was observed by higher concentration (10000 µg/ml) that gave no growth for 17 isolates from 20, these results were observed by nacked eye, while lower effect by low concentration (5000 and 2500 µg/ml) that gave no growth for 2 and 1 isolates respectively, as show in Figure (2). The area of concern is that MIC values of the active plant extracts obtained in this study, suggesting that the plant extracts were bacteriostatic at lower concentration.

The results presented in this report highlight the potential of rosemary extract as a source of antibiotic resistance modifying compounds. Sandasi *et al*, (20) reported that the best activities comparable to ciprofloxacin (75%) were *Rosmarinus officinalis* (78%) and other plant extract and this result was in agreement with present study.

The antimicrobial actions of essential oils inhibited the common biochemical pathways involved; inactivate microbial enzyme [21], rupturing of cell membrane and cause increase membrane permeability [22, 23]. Essential oils may interrupt the constitution of different fatty acids, polysaccharides and phospholipids layers find in the cell wall and plasmaic membrane [24]. Essential oils can cause membrane disruption in microorganisms by the action of lipophilic compounds in the oils and thus imparting antibacterial effect [25].

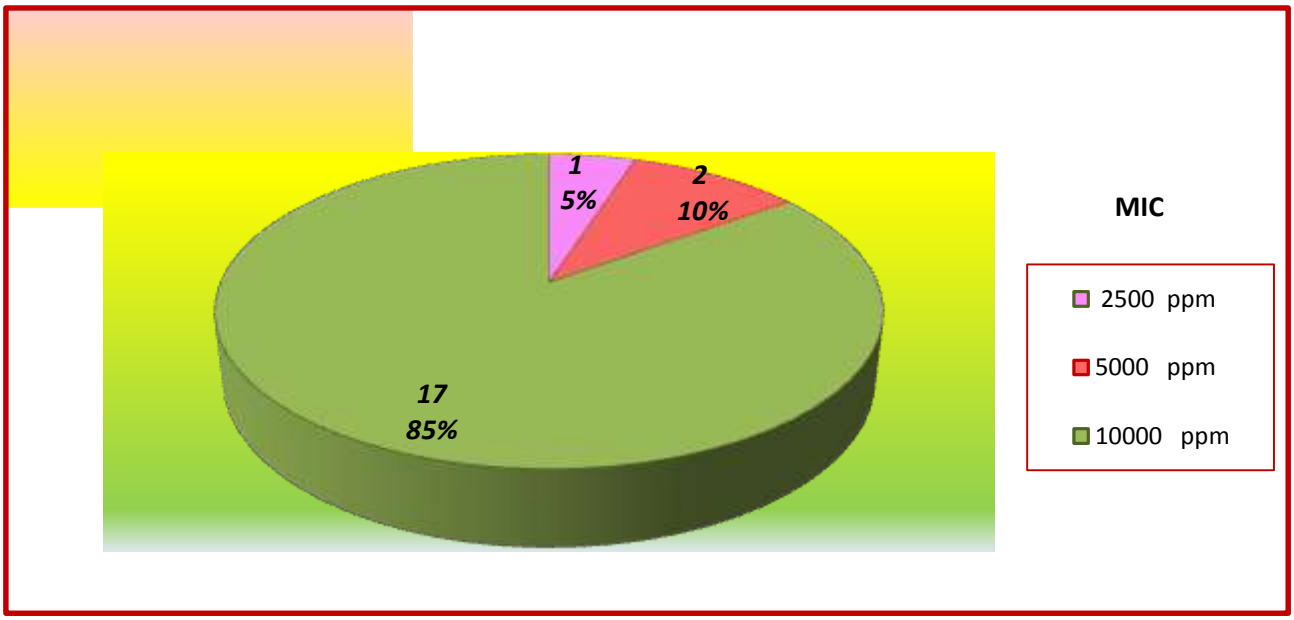

Figure 2: Effect of *Rosmarinus officinalis* essential oil on planktonic cells

In Vitro the Synergistic Inhibitory Activity of *C. citrates* and R. *officianalis* on planktonic *S. aureus*

In other experiment, study the combination effect of *C. citrates and R. officianalis* on planktonic *S. aureus* was done, the results showed that the antibacterial effects by two essential oil together were better than from use each one of them only. Thirteen bacterial isolates was inhibited by concentration (5000 µg/ml) in percentage (65%), the other effects against seven isolates with percentage (35%), see Figure (3). In the present study, rosemary exhibits remarkable synergistic activity in combination with *C. citrates*, which is reflected by changes in the MIC values of the test MRSAs. The results seem promising considering that crude extracts were used. The potentiation is likely to have been much more pronounced if pure compounds were used. Nguefack *et al* [26]. Found synergistic activity of essential oil fractions from *C. citratus*, *Thymus vulgaris* and *Ocimum gratissimum* against *Penicillium expansum*. The results reported that4 mixtures among 23 mixtures of essential oil fractions tested, gave synergistic effect. These combinations increased the antimicrobial effect and reduced the amount of essential oil necessary for antimicrobial activity.

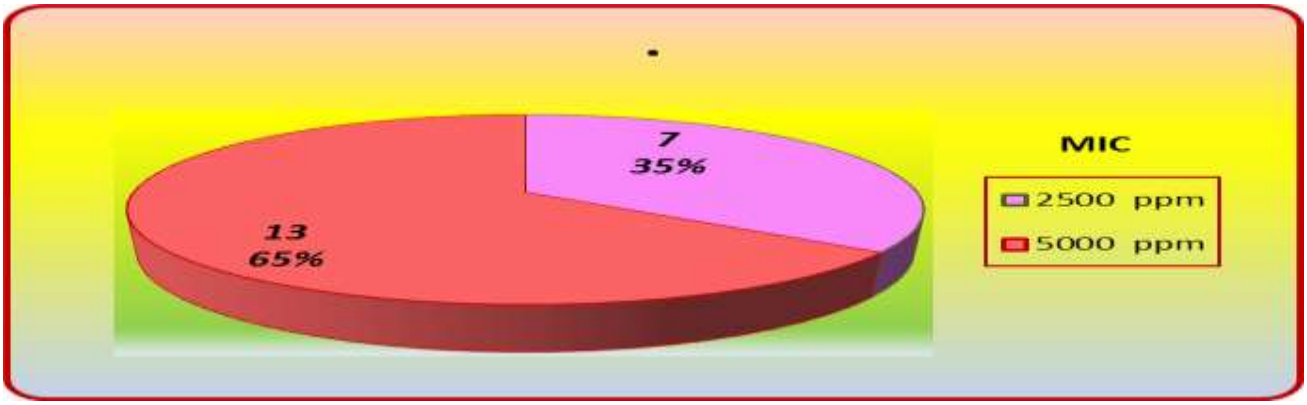

Figure 3: The synergistic effect of Rosmarinus *officinalis* and Cymbopogon *citratus* against planktonic cells

When compared the effect of each one of plant oils alone and when synergism between them was made, it was observed that the synergistic effect gave highly effect against planktonic cells of bacteria in concentration 5000 µg/ml and 2500 µg/ml respectively, while when use each plant oil alone the highly effect achieved in concentration 10000 µg/ml (see Figure (4).The results obtained from broth dilution method support the general indication that gram positive organisms are more sensitive to the oil. Similar observations were made by Onawunmi and Ongulana [27] and Cimanga et al [28].

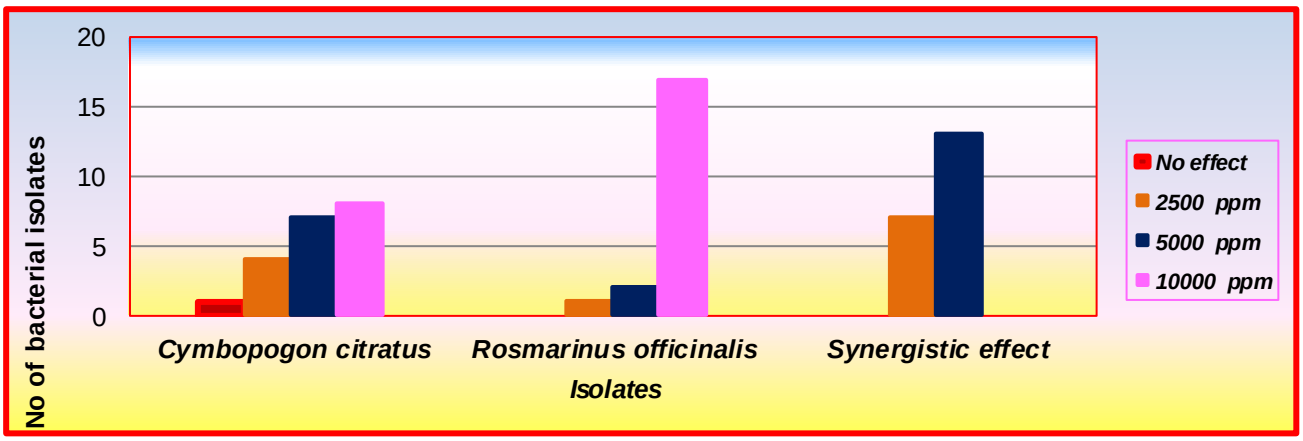

Figure 4: Comparision between the effect of *Rosmarinus officinalis* and Cymbopogon *citratus* and synergistic against planktonic cells

Biofilm Assay

S. aureus Biofilm Formation

Test strains of *S. aureus* were investigated for their biofilm forming ability. Fifteen strains formed strong biofilm in percentage (75%), four formed moderate biofilm (20%) and one strain formed weak (5%) as determined by crystal violet staining assay using tissue culture plate method (Table (1). These strains were divided as strong (OD 490 > 4* control), moderate (OD 490> 2* control) and weak (OD 490 < 2* control) biofilm former based on their absorbance in crystal violet assay (Table 4). Strains visually exhibiting strong biofilm showed absorbance values > 0.3 when assessed by crystal violet assay in 96 well microtitre plates.

Table 1: Biofilm producing by *S. aureus* before treatment, using microtiter plate assay

Isolates	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
O.D	1.759	0.275	0.155	0.444	0.434	**0.210**	0.476	0.416	**0.616**	**0.165**
Biofilm producing	Strong	Moderate	Weak	Strong	Strong	Moderate	Strong	Strong	Strong	Moderate
Isolates	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
O.D	1.864	3.137	0.446	0.847	1.405	1.544	1.261	0.299	0.486	1.648
Biofilm producing	Strong	Strong	Strong	Strong	Strong	Strong	Strong	Moderate	Strong	Strong

The differences in biofilm thickness resulted from different reasons such as differences in isolates capacity to form biofilm. Perhaps, the primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (quorum sensing signaling molecules) that produced from each isolate play an essential role [29, 30]. Biofilm resistance to antibiotics necessitates new measures for the management of the infections produced by the responsible biofilms [31].

Biofilm and multidrug resistance has been identified as virulence factors of great magnitude in clinical infections. Due to the increase in complexity of most microbial infections and the resistance to conventional therapy, researchers have been prompted to identify alternatives for the treatment of infections. Plant extracts and other biologically active compounds isolated from plants have gained widespread interest in this regard as they have been known to cure diseases and illness since ancient times. Gutierrez *et al* [32]. Demonstrated biofilm forming ability of 63 S. aureus isolates isolated from 442 environmental samples on congo red plates and by PCR amplification of genes involved in biofilm formation. Li *et al* [33]. In his study showed O33 stain to be high biofilm producer by both tetrazolium reduction assay and by crystal violet assay and stated crystal violet staining method to be accurate and faster. Saxena *et al* [34].Reported modified tissue culture plate method to be accurate screening method as compared to other methods for studying biofilm formations.

The Antibiofilm Activity of *C. citratus* on MRSA

C. citratus gave antimicrobial effect against planktonic form of MRSA in all its concentrations, depending on these results the antibiofilm activity for this oil was done by using tissue culture plate method (TCP). There are little studies about the effect of *C. citratus* on bacterial biofilm, while several studies conducted on its effect against bacteria in a planktonic form. The present research interested in study of effect of this oil on MRSA biofilm by tissue culture plate method, the results indicated that *C. citratus* was effective against 15 bacterial isolates (75%) and only 5 isolates not affected see Figure (5).

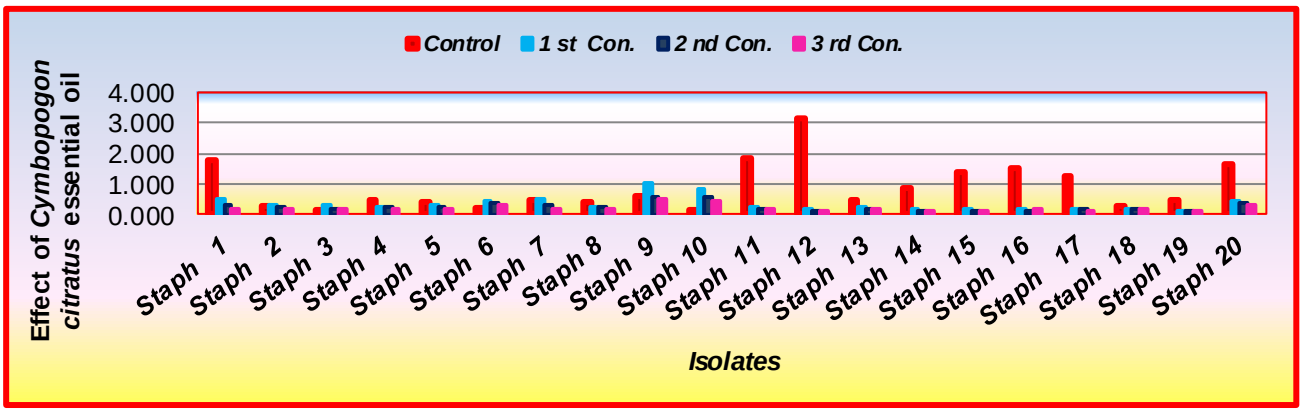

Figure 5: The antibiofilm activity of *C. citratus* on MRSA

Adukwu *et al* [35]. Reported in their study the anti-biofilm properties of lemongrass against *S. aureus* biofilms. Where previously Bearden *et al.* (36), investigated commercial formulations containing essential oils against MRSA, this is first study that has demonstrated the anti-biofilm activity of lemongrass essential oils against biofilms of methicillin sensitive *S. aureus* (MSSA) and MRSA and this was in agreement with current study. Further studies would be required to evaluate any potentially toxic effect of lemongrass essential oils; however, the antimicrobial and antibiofilm properties provide another option for future antimicrobial therapeutic interventions in both clinical and industrial applications.

The antibiofilm activity of *R. officianalis on* MRSA

Tissue culture plate method by another study was used to investigate the antibiofilm activity of *R. officianalis* against MRSA depending on the effects of it on planktonic cell for these bacteria. The results of present study revealed that *R. officianalis* were affected against 17 bacterial isolates (85%)

but there was no effect against 3 isolates (15%), see Figure (6).

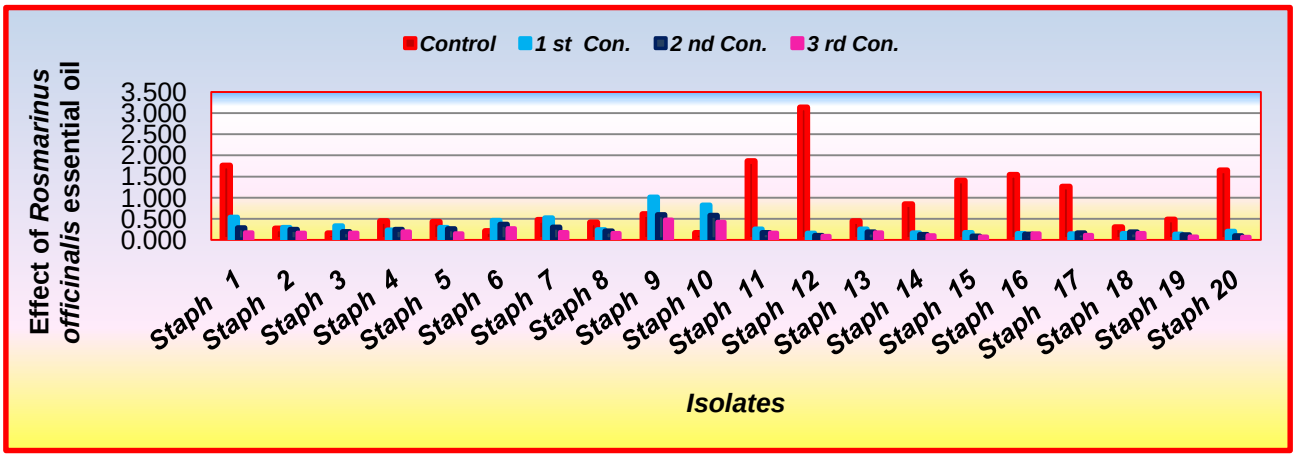

Figure 6: the antibiofilm activity of *R. officianalis* on MRSA

The result of present study was in agreement with the result of Freire *et al.*(37) When they compare the extracts used, *R. officinalis* presented the best antibiofilm effect, and mention that *R. officinalis* extract utilized in their study promoted reductions ranging from 12.1% to 78.7% in biofilms formed by isolates of coagulase-positive staphylococci. Other study reported that R. officinalis extract presented more than 50% of antibiofilm activity. *S. aureus* is an opportunistic pathogenic microorganism that has developed antibiotic resistance to penicillin by betalactamase plasmid, and causes a wide range of infections, including acute, chronic, and toxin mediated disease [38].

The Synergistic Antibiofilm Activity of *C. citrates* and *R. officianalis* on MRSA

The minimum inhibitory concentration (MIC) that affect on planktonic cells was used against biofilm, the same protocol in biofilm assay was used on microtiter plate method but after biofilm formation the plant oil was added before staining step. However, both *C. citrates and R. officianalis* exhibited high activities against most MRSA bacteria in 85% from bacterial isolates (Figure (7). Only 3 isolates not affected by these oils together and represent (15%) from all isolates. when compare the results of this experiment with the previous study when each one of plant oil alone, it was observed that the synergistic effect against bacterial isolates was higher.

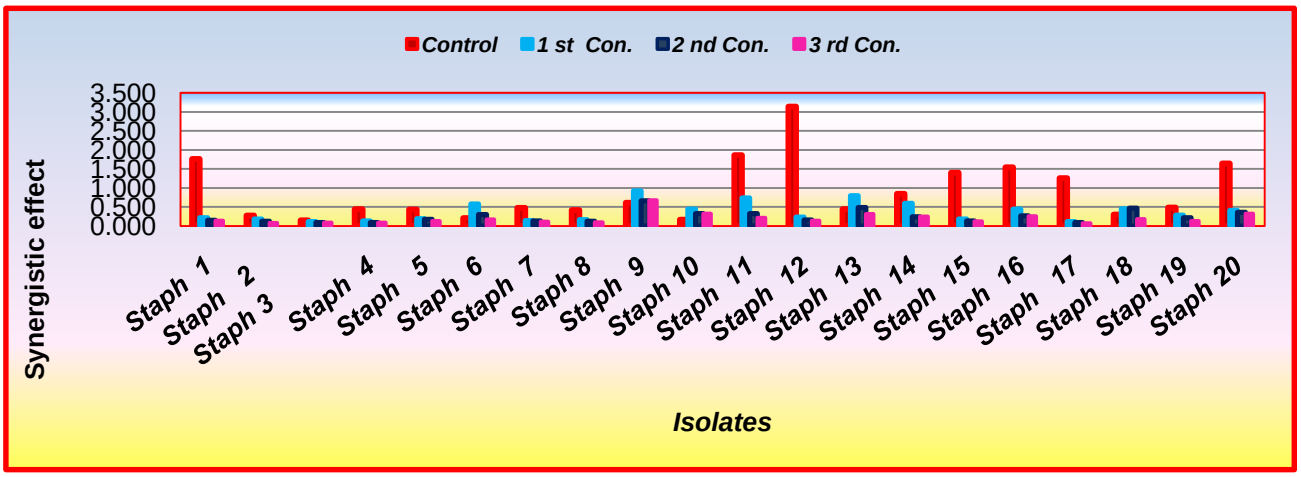

Figure 7: the synergistic antibiofilm activity of *C. citrates* and *R. officianalis* on MRSA

The use of essential oils as function of ingredients in foods, drinks and cosmetics is gaining force, both for the rising interest of consumers in use of natural ingredients and increasing concern about potentially unsafe of synthetic additives [39]. Essential oils are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries [40]. Essential oils exhibit various biological activities like antibacterial [41], antifungal antiviral, insecticidal, antioxidant, anticancer activity, anti-inflammatory, Anti-staphylococcal activity, Antimycotic activities

and anti-diabetic, etc. Some oils are also used in food preservation, aromatherapy and fragrance industries [42, 43, 44, 45].

Conclusion

Based on these data, the potential use of these essential oils is promising, since demonstration of in vitro antimicrobial activity is the first step in the regulatory process: This study shows that the synergistic effect between both lemon grass and rosemary essential oils can inhibit the growth of MRSA, a very significant public health concern. The comparative effects of lemongrass oil and rosmarry against test organisms are demonstrable indications of the oil as an antibacterial agent. Thus, we conclude that in present era of emerging multidrug resistance among gram positive organism's lemongrass oil will be helpful in treating such infections. If further studies definitely show safety and efficiency of these essential oils, this may represent a valuable weapon against MRSA.

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