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THE ANTIMICROBIAL AND ANTIBIOFILM EFFECTS OF *CITRUS LIMON* AND *CINNAMON CASSIA* ESSENTIAL OILS AGAINST BIOFILM PRODUCING *STAPHYLOCOCCI* CAUSING CHRONIC TONSILLITIS

Elana Rafat Zaky[1*], Nahla M Elsherbiny[2], Soad Abdel latief Hassan Bayoumi[3], Mohamed M Osman[4], Amany M Nafe[2], Rania Bakry[5], Mohamed Saad Badary[2]

[1]*Microbiology and Immunology department, Faculty of Pharmacy, Assiut University, Egypt*
[2]*Medical Microbiology and Immunology Department, Faculty of Medicine, Assiut University, Egypt*
[3]*Pharmacognosy department faculty of pharmacy assiut University, Egypt*
[4]*Otorhinolaryngology & Head and Neck Surgery Department, Faculty of Medicine, Assiut University, Egypt*
[5]*Clinical Pathology Department, South Egypt Cancer Institute, Assiut University*

Objectives: *The objectives of this study were to identify biofilm producing Staphylococci causing chronic tonsillitis, to extract Citrus limon and Cinnamon cassia essential oils and to study their antimicrobial and antibiofilm properties.* ***Patients and Methods:*** *Tonsillar biopsies were collected from 163 chronic tonsillitis patients undergoing tonsillectomy at the Otorhinolaryngology & Head and Neck Surgery Department, Faculty of Medicine, Assiut University. The isolates were microbiologically identified, and theantimicrobial sensitivity pattern was determined. Extraction of essential oils (EOs) fromfresh citrus limon fruits peel (CEO) and dry Cinnamon cassia (CCEO) was done by Hydro distillation and were characterized by Gas Chromatography-Mass Spectrometry. The MIC, MBC, MBEC and MBICof the extracted oils were determined. Scanning Electron Microscopy (SEM) analysis was done before and aftertreatment with EOs and vancomycin.* ***Results:*** *Bacteria were isolated from 75 tonsillar specimens, giving a total percent of 46.01% (75/163). Biofilm producing Staphylococcus aureus was the most common organism (77.3 %, 58/75). The highest antimicrobial sensitivity was for vancomycin (90.6%) and erythromycin (81.3%). For CEO, the MIC, MBC, MBIC and MBEC values ranged from 0.25 to 4% v/v, while for CCEO (0.625 to 10% v/v). By SEM analysis, damage of S. aureus cells occurred by the effect of EOs and vancomycin. The major components of CEOwere alpha-limonene (10.9%) and beta-I-pinene (10.7 %), while forCCEO, it was trans-cinnamic aldehyde (71%).* ***Conclusions:*** *Biofilm producing S. aureus is the most common bacteria causing chronic tonsillitis. CEO and CCEO could haveapotential prophylactic and therapeutic use against biofilm producing Staphylococci causing chronic tonsillitis.*

Keywords: *citrus essential oil; Cassia essential oil; Staphylococci; chronic tonsillitis*

INTRODUCTION

Recurrent and chronic tonsillitis involve repeated attacks of inflamed tonsils which may have a great impact on the patient's quality of life[1,2]. Although many studies discussedthe bacterial causes of recurrent tonsillitis, yet it still remains a controversial issue[2]. The bacteriological profile of chronic tonsillitis is not constant because of the pathological changes that occur in chronic tonsillitis, the misuse of antibiotics, the resistance of the

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*Corresponding author: Elana Rafat Zaky, E-mail: elana_raafat@hotmail.com

causative bacteria and the difference in the bacteria isolated from the tonsillar surface (by swabbing) from those obtained from the tonsillar core (by biopsy) [2]. Bacteria in the tonsillar core play a vital role in the recurrent nature of infection [3].

Bacteria causing chronic tonsillitis generally differ from those causing acute tonsillitis by being resistant to common antimicrobials or by their ability to create biofilms in the warm and wet folds of the tonsils which act as a continuous source of infection[4, 5].

Bacterial biofilms are communities of microbial cells enclosed in a three- dimensional structure of self- produced extracellular polymeric matrix (EPS), composed of exopolysaccharides, proteins, DNA, and water. This protected organization of the biofilm guarantees the resistance to host's immune system, the exchange of genetic material, the enhanced antimicrobial resistance and thus bacterial survival in unfavorable environments [6]. Bacteria within biofilms can tolerate levels of antimicrobial agents 1,000 times higher than when they are present in the planktonic form. In the EPS, subpopulations of bacterial cells, known as 'persisters', can tolerateantibiotics, survive and even repopulate the biofilm periodically when the concentration of antibiotics reduces leading to the release bacteria in the planktonic form from the biofilm[7].

The process of biofilm formation is triggered with the adherence of planktonic microorganisms to surfaces followed by multiplication and aggregation within self-produced extracellular polymeric substances (EPS) leading to the microcolony formation. A mature biofilm is a three-layered structure: inner regulating layer, middle microbial basement layer, and outer layer inhabited by the planktonic form of microorganisms that are ready to form the biofilm. Finally, matured biofilm ruptures to disperse the microorganisms to begin a new cycle of biofilm formation [8]. In *S.aureus*, interactions with abiotic hydrophilic surfaces are controlled by polysaccharide intracellular adhesion (PIA), which is encoded by the ica operon (icaABCD)[9].

Despite recent scientific advances in pharmacotherapy, yet the prevalence of chronic tonsillitis remains high due to antibiotic abuse, misuse and biofilm production thus necessitating the need foreffective non-toxic natural antibacterial agents in the quest for new prophylactic and therapeutic agents that might control infections without emerging resistant bacterial strains[10].

Establishing efficient methods to combat bacterial biofilms is a major concern. Natural compounds, such as essential oils derived from plants, are among the favored and recommended strategies for combatting bacteria and their biofilm [11].

Essential oils (EOs) are natural products that contain volatile organic compounds which are synthesized as a result of secondary metabolism of aromatic plants[12, 13]. These oils have many antibacterial, antibiofilm, anti-fungal, antiviral and antioxidant, activities [14]. The great advantage of EOs is that their usage is not likely to select for microbial resistance because they have acomplex composition and, therefore, multiple targets in the microbial cells. *Citrus limon* and *Cinnamon cassia* essential oils have manyantimicrobial and antibiofilm effects against various microorganisms[15].

The objectives of this study were to identify and characterize biofilm producing *Staphylococci* causing chronic tonsillitis in our community, to extract and define the chemical composition of *Citrus limon* and *Cinnamon cassia* essential oils and to study their antimicrobial and antibiofilm propertieson the clinical isolates and on the *Staphylococci* reference strain ATCC 25923.

PATIENTS AND METHODS

Sample collection

Thiscross-sectionalstudy was carried out during a period of 24 months from May 2021 to May 2023. Tonsillar biopsies were collected from 163 patients suffering from chronic tonsillitis who were admitted to theOtorhinolaryngology & Head and Neck SurgeryDepartment, Faculty of Medicine, Assiut University Hospital for tonsillectomy, after taking the consent from the patients or their guardians depending on their age. This work was approved by the Ethical Committee of the Faculty of Medicine, Assiut University (IRB:04-2023-200150).

Inclusion criteria: patients undergoing tonsillectomy with > 4 attacks oftonsillitis attacks per year.

Exclusion criteria: patients who had recently taken antibiotics within 2 weeks were excluded from the study.

The tonsils were dissected to expose the core. All samples were placed in tryptic soya broth (tryptone soy broth) and incubated for 24h before examination. Then samples were cultured on Columbia blood agar plates using 10% defibrinated sheep blood, in the presence of 5-10% CO_2 and blood agar in ambient air for 24h at 37 °Cfor aerobic and facultative anaerobic bacteria. The isolates were microbiologically identified and were confirmed by VITEK 2 system (DensiChek; bioMérieux) (software version 4.01).

Antimicrobial sensitivity testing

For *Staphylococci* isolates, antibiotic sensitivity tests were done and interpreted as per CLSI (2020) using disk diffusion method on Muller–Hinton agar using the following antibiotics:

Vancomycin 30 (VA30), Co-Trimoxazole 25(COT) (Trimethoprim /Sulfamethoxazole), Ceftriaxone 30(CTR), Tetracycline 30 (TE), Levofloxacin 5(LE), Erythromycin 15(E), Amoxyclav 30 (AMC) (Amoxicillin /Clavulanic acid). [16].

Detection of biofilm formation

Tissue culture plate method

A single colony from blood agar was inoculated into a glass tube containing two ml tryptone soy broth with 1% glucose (TSBglu). The tubes were incubated overnight at 37°C under aerobic conditions then 200 ml were aseptically transferred in the wells of a flat-bottomed micro wellplastic plate that was incubated overnight at 37°C without sealing of the plate for proper oxygenation. Next day, the contents were discarded, and the micro well plastic plate was washed once by adding 200 µl PBS (pH 7.2) into each well and then discarded followed by biofilm fixation by adding 200ul of freshly prepared sodium acetate (2%) for 10 minutes and then discarded. This was followed by biofilm staining by adding 200 µl crystal violet (0.1%) to each well. The Plates were kept at room temperature for 30 minutes, and then the stain was discarded. The washing step was repeated once more. Finally, the plate was left to dry at room temperature for one hour, after which, the absorbance was read on a spectrophotometer at 620 nm OD. The results were interpreted as: <0.120 for non-biofilm producers, 0.120-0.240 for moderate biofilm producers and >0.240for strong biofilm producers[17].

The Congo red method

Staphylococcal strains were inoculated on the prepared media and incubated aerobically at 37°C for 24 hours. Black colonies with dry crystalline consistency indicated biofilm formation. Red colonies with occasional darkening at the center of the colonies were considered non-biofilm producers [18].

Extraction and characterization of essential oils

Plant material

The plant material used in this study were fresh*citrus limon* fruits peel and dry *Cinnamon cassia* bark that were purchased from the Egyptian markets.

Extraction of the essential oils

Hydro distillation method with the use of a Clevenger apparatus was used. Samples were separately weighed and placed in one-liter rounded flask and connected to the Clevenger apparatus. Five hundred ml of distilled water was added to the flask and heated to the boiling point. The steam in combination with the essential oils were distilled into a graduated cylinder for 7 hours and then separated from aqueous layer. Oil samples were collected and stored in vials at -18 °C until use[19, 20].

Identification of the essential oils by Gas Chromatography-Mass Spectrometry

The GC- MS analysis was performed as described previously. The oil components were identified by comparison of their retention times and mass spectral data with mass spectra library[21].

Antimicrobial and antibiofilm activity of the extracted oils

The minimum inhibitory and bactericidal concentrations, minimum biofilm inhibition and eradication concentrations, were determined for the essential oils for all

staphylococcal isolates in addition to the reference strain*Staphylococcus aureus*ATCC25923. The analysis of each was performed in triplicates.

Determination of minimum inhibitory concentration (MIC) of the essential oilsbybroth microdilution method

MICs were determined in 96-microtitration well plates. The essential oil (EO) was diluted in Tryptic Soya broth (TSB) with 20% dimethyl sulfoxide (DMSO), starting from the 4% concentration (v/v) down to 0.03% (v/v) in final volume for the citrusessential oil (CEO) and 10% concentration (v/v) down to 0.156 % (v/v) for the *Cinnamon cassia* essential oil (CCEO). Each solution was tested in triplicate. Negative (TSB broth with DMSO: Distilled water) and positive (TSB broth and bacterial inoculum, without EO) controls were prepared for each plate. One hundred µl of TSB was poured into the wells and then 100 µl of essential oil was added to the first well of each row. After mixing the contents of the first well, 100 µl of it was removed and added to the next well and so on, then 100 µlwas discarded from the final well. Then 100 µl of the bacterial suspension (0.5 McFarland turbidity standard providing an optical density comparable to the density of a bacterial suspension with a 1.5 x 10^8 colony forming units (CFU/ml)) was added to each well. The plates were incubated at 37 °C for 24 h. The MIC value was determined as the lowest dilution where no bacterial growth was observed[22-24].

Determination of the minimum bactericidal concentration (MBC)

A total of 20 µL from clear wells of the MICs test were plated on blood agar plates and were incubated at 37 °C for 24 h. MBC values were defined as the lowest concentration of the sample which resulted in ≥99.9% kill of the initial inoculums [25].

Determination of the minimum biofilm eradication concentration (MBEC)

Biofilm formation was done by adding 100µl of the bacterial suspension to each well then incubated for 24hr. Then, the medium was discarded, and the wells gentlyrinsed twice with PBS followed by the addition of 100 µl of the Eos which were serially diluted into the wells. The plates were then incubated for 24 h at 37°C then the contents of the wells were decanted, and each well was gently rinsed twice with 300 µl of sterile phosphate buffered saline (PBS) (pH: 73 ± 03). The plates were air dried for 30 min, stained with 1% (w/v) crystal violet for 30 min at room temperature, washed three times with PBS (200 µl per well) and dried. The crystal violet was then solubilized using 10% (v/v) glacial acetic acid and the OD measured at 595 nm using a Microplate reader (Bio-Rad 680XR). The MBEC was determined as the EO concentration at which the OD< negative control. It is the lowest concentration of the antimicrobial agent that eradicates already formed biofilm [26].

Determination of the minimum biofilm inhibitory concentration (MBIC)

The EOs was tested for their potential to prevent biofilm formation. The same steps used in MIC determination were done, where 100µlof bacterial suspension (0.5 McFarland turbidity standard) was added in addition to the EOs emulsified in TSB with 20% DMSO that were serially diluted as mentioned previously and were added to the U- bottomed 96-well microtiter plate. Positive and negative controls were included. The final volume was 200 µl in each well. The analysis was performed in triplicates. After incubation at 37°C for 24 h, the biofilm was measured by crystal violet as previously described [27].

Antimicrobial activity of vancomycin

Determination of minimum inhibitory concentration of vancomycin

The MIC value was determined for the clinical isolates and the reference strain according to the CLSI 2020 guidelines[28].

Scanning Electron Microscopy (SEM) Analysis

The *Staphylococcus aureus* ATCC25923 strain suspension (10^8 CFU/mL) was either untreated or treated with CEO or CCEO or vancomycin at the MIC values and incubated at 37 °C for 24h. Thereafter, the suspensions were washed with PBS and centrifuged at 5000× g for 10 min at 4 °C, and the precipitated cells were fixed in 2.5% glutaraldehyde at 4 °C for 6 h. Subsequently, the fixed cells were

dehydrated with a series of differentconcentrations (25%, 50%, 75%, 95%, and 100%) of ethanol for 10 min. Finally, the dehydrated samples were coated with gold, and observed by a SEM [29].

Statistical analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS) software program (version 26.0). Qualitative variables were recorded as frequencies and percentages. Quantitative measures were presented as means ± standard deviation (SD).

RESULTS AND DISCUSSION

Results

Demographic data of the patients

The current study included 163 patients: 90 females and 73 males. The age of the patients included in the study ranged from 3-20 years withmean±SD 9.5±2.81. The number of attacks of tonsillitis ranged from 4 to 8 attacks per yearprecedingtonsillectomy.

Microbiological causes chronic tonsillitis

Aerobic bacteria were isolated from 75 tonsillar specimens of patients with chronic tonsillitis undergoing tonsillectomy, giving a total percent of 46.01% (75/163). Among the isolated aerobic bacteria, *Staphylococcus aureus* was the most common organism (77.3 %, 58/75) causing chronic tonsillitis followed by coagulase negative staphylococci (8 %, 6/75) as shown in **Table (1)**.

Antimicrobial susceptibility of the isolated *Staphylococci*

The highest sensitivity was for vancomycin (90.6%) followed by erythromycin (81.3%) and the least sensitivity was for levofloxacin (45.3%) as shown in **Table (2)**.

Detection of biofilm formation

All *staphylococcal* isolates were biofilm producers, with 62.5% (40/64) being moderate producers and 37.5% (24/64) being strong producers.

Table 1: Aerobic bacteria isolated from tonsillar biopsies of patients with chronic tonsillitis undergoing tonsillectomy.

Aerobic isolates	No. (%)
Gram positive cocci	
Staphylococcus aureus	58(77.3%)
Staphylococcus lentus	3(4%)
Staphylococcus chromogenes	1(1.3%)
Staphylococcus haemolyticus	1(1.3%)
Staphylococcus pseudintermedius	1(1.3%)
Pneumococcus	8(10.7%)
Kocuriakristinae	1(1.3%)
Gram negative bacilli	2(2.6%)
Total	**75 (100%)**

Table 2: The antimicrobial sensitivity pattern of the isolated *Staphylococci*.

Antibiotics	Staphylococcus (n=64)		
	S	I	R
	No. (%)	No. (%)	No. (%)
Vancomycin 30 (VA30)	58(90.6%)	2(3.1%)	4(6.3%)
Trimethoprim/Sulfamethoxazole 25 (COT)	51(79.7%)	7(10.9%)	6(9.4%)
Ceftriaxone 30 (CTR)	32(50%)	24(37.5%)	8(12.5%)
Tetracycline 30 (TE)	48(75%)	12(18.8%)	4(6.3%)
Levofloxacin 5 (LE)	29(45.3%)	28(43.8%)	7(10.9%)
Erythromycin 15 (E)	52(81.3%)	10(15.6%)	2(3.1%)
Amoxicilin/Clavulanic acid 30 (AMC)	15(23.4%)	28(43.8%)	21(32.8%)

Antimicrobial activity of the extracted oils

Determination of MIC, MBC, MBEC and MBIC

Controls showed that the DMSO used was not responsible for the inhibitory effects. The MIC, MBC, MBIC & MBEC for the isolated *Staphylococci* using CEO are shown in **Fig. (1) and Table (3)** and those for CCEO are shown in **Fig. (3) and Table (4)**. The values for the *Staphylococci* reference strain ATCC 25923 using CEO were 0.5, 1, 0.5 and 2% v/v respectively and the values using CCEO were 2.5%, 5%, 5 %and 10 % v/v respectively. The values of the oils on the vancomyc in resistant strains are shown in **Tables (5-6).**

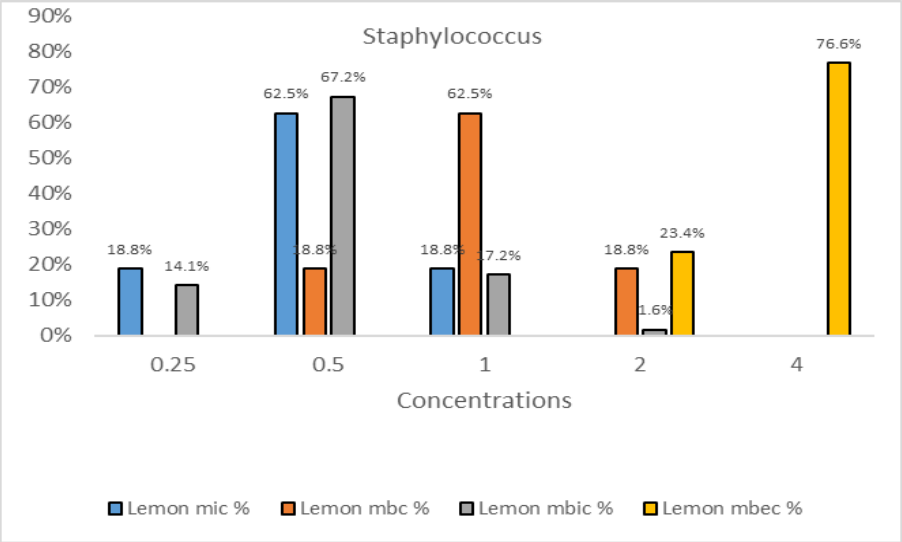

Fig. 1: The MIC, MBC, MBIC and MBEC values of Lemon oil against *Staphylococci* isolates.

Table 3: The MIC, MBC, MBIC and MBEC values of CEO against isolated *Staphylococci*.

Concentrations	***Staphylococci* isolates (n=64)**			
	CEO			
	MIC N (%)	**MBC N (%)**	**MBIC N (%)**	**MBEC N (%)**
0.250	12(18.8%)	-	9(14.1%)	-
0.500	40(62.5%)	12(18.8%)	43(67.2%)	-
1	12(18.8%)	40(62.5%)	11(17.2%)	-
2	-	12(18.8%)	1(1.6%)	15(23.4%)
4	-	-	-	49(76.6%)

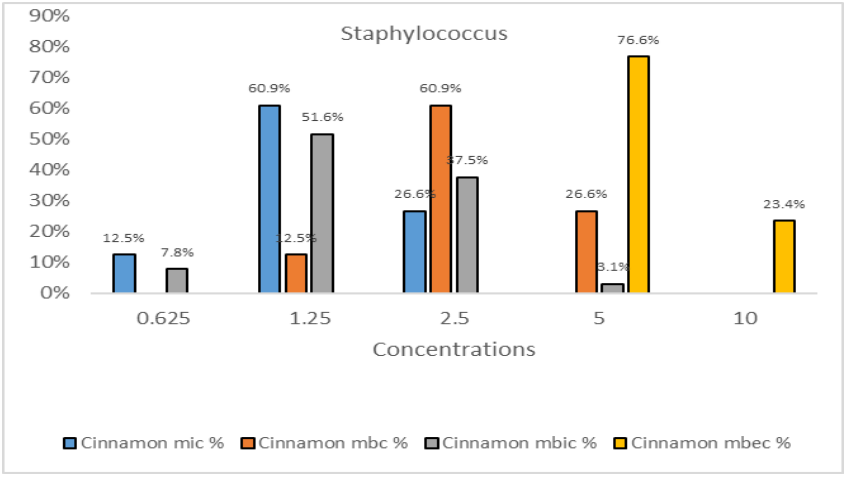

Fig. 3: The MIC, MBC, MBIC and MBEC values of CCEO against isolated *Staphylococci*.

Table 4: The MIC, MBC, MBIC and MBEC values of CCEO against isolated *Staphylococci*.

Concentrations	Staphylococci isolates (n=64)			
	CCEO			
	MIC N (%)	MBC N (%)	MBIC N (%)	MBEC N (%)
0.625	8(12.5%)	-	5(7.8%)	-
1.250	39(60.9%)	8(12.5%)	33(51.6%)	-
2.5	17(26.6%)	39(60.9%)	24(37.5%)	-
5	-	17(26.6%)	2(3.1%)	49(76.6%)
10	-	-	-	15(23.4%)

Table 5: The MIC, MBC, MBIC and MBEC values of Lemon EO against vancomycin resistant *Staphylococci.*

	Lemon EO			
	MIC	MBC	MBIC	MBEC
	Vancomycin resistant isolates			
	R (N=6)	R (N=6)	R (N=6)	R (N=6)
0.5	1 (16.7%)	-	1 (16.7%)	-
1	5(53.3%)	1 (16.7%)	5 (83.3%)	-
2	-	5 (83.3%)	-	-
4	-	-	-	6 (100%)

Table 6: The MIC, MBC, MBIC and MBEC values of Cassia EO against vancomycin resistant *Staphylococci.*

	Cassia EO			
	MIC	MBC	MBIC	MBEC
	Vancomycin resistant isolates			
	R (N=6)	R (N=6)	R (N=6)	R (N=6)
1.25	3 (50%)	-	3 (50%)	-
2.5	3 (50%)	3 (50%)	3 (50%)	-
5	-	3 (50%)	-	4 (66.7%)
10	-	-	-	2 (33.3%)

Determination of MIC of vancomycin

All the vancomycin resistant isolates by disc diffusion (n=4) had an MIC of 8µg/ml and the vancomycin MIC for those with intermediate vancomycin resistance by disc diffusion (n=2), was 4µg/ml. The MIC for the isolatesthat were vancomycin susceptible by disc diffusion, was 2 µg/ml. For the *Staphylococcus aureus* ATCC 25923, the vancomycin MIC was 2µg/ml.

Scanning Electron Microscopy (SEM) Analysis

The *Staphylococcusaureus*ATCC 25923strain had a normal regularspherical shape with an intact membrane andretained normal cell morphology and inner structure as shown in **Fig. (4A)**.

After treatment with CEO and CCEDat the MIC values, damage had formed on the surface of cells related with obvious distortion of *S. aureus* cells, with an unclear profile, collapsed surface and damaged cell membranewith losses of intracellular dense vital constituents as shown in **Fig.s (4B&4C)**. Vancomycin treatment at the MIC value led the cells to lyse, become irregular, smaller, variable in size and adhesive to each other as shown in **Fig. (4D)**.

Preparation and characterization of the essential oils

Extraction of essential oils by hydro-distillation of the plants by using Clevenger type apparatus.

Each 300g of *Citrus Limon* gave 2ml lemon oil and each 200g of *Cinnamon Cassia* gave 2 ml cassia oil as shown in **Table (7)**.

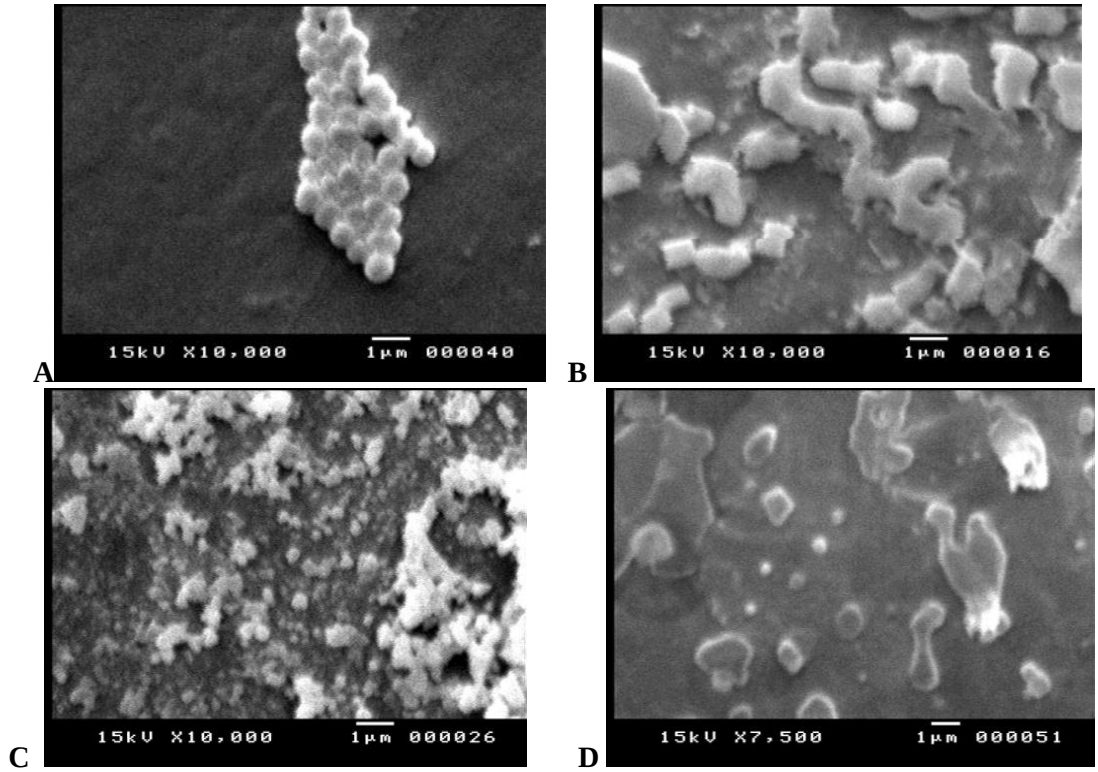

Fig 4: Scanning electron micrographs of *Staphylococcusaureus* ATCC 25923 strain (A untreated, B treated with Lemon EO at 0.5% v/v (MIC), C treated with Cassia EO at 2.5% v/v (MIC) and D treated with vancomycin at2%v/v (MIC).

Table 7: Extraction of essential oils by hydro-distillation of the plants.

Plant name	Weight (kg)	Volume (ml)	Yield %(v/w)
Citrus Limon	1.5	10	0.66
Cinnamon Cassia	0.8	8	1

Identification of the components of the essential oils by Gas chromatography mass spectrometry (GC-MS)

Analysis of essential oils showed that the major components of *Citrus Limon*oil were alpha-limonene (10.9%), beta pinene (10.7 %), alpha pinene (6.5) and gamma terpinen (6.1%). While the major components of *Cinnamon Cassia* oil were trans-cinnamic aldehyde (71%), delta-cadinene (5.2%) and alpha-muurolene (4.8%).

Discussion

Untreated or unproperly treated tonsillitis in children may lead to many complications therefore, identification of bacterial isolates and determination of suitable treatment is very important[30]. The bacteriological profile of chronic tonsillitis undergoes continuous changes [31], and differs from those causing acute tonsillitisby their antimicrobial resistance or by their ability to form biofilms [2, 31].

The current study included 163 patients aged 3-20 years (mean±SD 9.5±2.81) with chronic tonsillitis (4 to 8 attacks per year) from whom tonsillar specimens were taken at the time of tonsillectomy for microbiological diagnosis. Aerobic bacteria were isolated from 75 tonsillar specimens, representing 46.01% (75/163). The percent of bacterial isolation differed according tothe specimen and media used. It was reported to be 34.2% in throat swabs in the study of Abd El Galil et al and 50% in the study of Kurien et al,. In a recent study, a positive bacterial growth was 60.6% (60/128) among tonsillar crypts taken with a punch- biopsy needle preoperativelyfrom patients with repeated tonsillitis[32]. This study didn't cover the anaerobic or fungal causes.

Among the isolated aerobic bacteria, *Staphylococcus aureus* was the most common organism (77.3%, 58/75) causing chronic tonsillitis followed by coagulase negative *Staphylococci* (8 %, 6/75). This is in general agreement with many studiesconcerning recurrent tonsillitis that reported *S. aureus* to be the commonest cause but with different percentages. Hussien et alin Egypt, reported*Staphylococcus aureus*in 40%[33] and Klagisa et al, reported it to be 33.3%. Also, Wang et al, isolated*S. aureus* from the tonsillar surface in 27.8% and from the core in 32.6% of recurrent tonsillitis patients. Also, El-Galil et al, reported*Staph aureus*to be the common isolate (17.1%) [34]. Amer et al, reported the percentage to be 86.4% from the tonsillar core of the patients [31]. In addition, Eid et al, showed that *S. aureus* (26%) followed by Coagulase negative *Staphylococci* (CONS)(17.4 %) were the etiological factors for chronic and recurrent tonsillitis[35]. This could be explained by the persistence of *S. aureus* in the tonsillar tissues due to antibiotic resistanceor to the fact that *S. aureus* is the leading Gram-positive bacteria found in the normal microbiota of oropharynx and nose[36].

In the current study we didn't isolate streptococci as a cause of chronic tonsillitis. Recently, the isolation rate of *Streptococcus* spp. was low in many studies. In a recent study, *Streptococci* were isolated in 17.2% (17 /99) of tonsillar biopsies from recurrent tonsillitis patients[32]. Other studies have also reported a low isolation rate (1.7–5%) withstreptococci being less prevalent in the tonsillar core (1.7%) as compared to the tonsillar surface in patients with recurrent tonsillitis (RT)[37, 38]. Thus it has been claimed that *S. pyogenes* in the RT pathogenesis has most likely been overrated or, alternatively, decreased in recent years [32, 39]. On the other hand, some studies reported *Streptococci*to be among the common causesof chronic tonsillitis [30, 40, 41]. These results suggest that variation in the method used for sample collection and the population contribute to the difference in the bacterial distribution.

All the *Staphylococci* isolated were biofilm producers by the microtiter plate method. In accordance to our results, the presence of biofilms was significantly higher in patients with recurrent tonsillitis (80%) as evidenced by scanning electron microscopy versuscontrols (45%) [42]. Recently, Klagisa et al, found that37.4% of recurrent tonsillitis (RT) cases were due to biofilm producers with a statistically significant association found between the presence of Gram-positive bacteria and a biofilm-formation phenotype in the RT group[32]. The role of *S. aureus* in the pathogenesis of RT exacerbationand in the resistance to antimicrobials is unclear because*S. aureus* isolated from patients with recurrent tonsillitis does not show a high antibacterial resistance. So, biofilm formation or other protective mechanisms is suggested to have a major role due to the localization of the bacteria in the biofilm leading to antimicrobial tolerance in spite of the absence of specific resistance mechanisms[39]. So, the main problem in the treatment of patients with RT is usually difficulty in the effective eradication of the pathogen rather than its antibiotic resistance.

The antimicrobial sensitivity pattern varied among different studies depending on many factors. In the present study, the highest sensitivity was observed to vancomycin (90.6 %) followed byerythromycin (81.3%). Many studies reported that vancomycin sensitivity was 100% among *S. aureus* isolates causing tonsillitis; in Egypt, Hussien et al, [33] and in China[43]. On the other hand, lower percentages of sensitivity to vancomycin and erythromycin (32.9% and 39.5% respectively) were reported in a recent Egyptian study [31]. Vancomycin and erythromycin sensitivity were reported to be 98% and 28.6% of planktonic *Staphylococci* isolates [44].

Our study reported that Trimethoprim/Sulfamethoxazole sensitivity was (79.7%) and levofloxacin sensitivity was 45.3%. These differed from the Egyptian study of Amer et al, who reported that19.7% of *the S.aureus* isolates causing chronic tonsillitis were sensitive to levofloxacin and 96.1% of *Staph* isolates wereresistant to trimethoprim /sulfamethazole[31]. On the contrary, De oliveira found the sensitivity to trimethoprim /sulfamethazole to be 91.4% for the *Staphylococci* isolates[44].

On the other hand, the lowest antibiotic sensitivity observed was for amoxicilin/clavulanic acid (23.4%) and ceftriaxone (50%). This may be due to the abuse and excessive use of cheap drugs, which can be afforded and administered without

culture diagnostic guidance in Egypt leading to increased resistance.

Although a wide range of antimicrobials have been produced in last years, yet unsuccessful antibiotic therapy is a common result which could be explained by low concentrations of the antibiotics in the tonsillar tissue, difficulty in identifying causative bacteria, biofilm producers or theantibiotic resistance patterns of the involved pathogenic bacteria [2, 45]. In traditional medicine, essential oils obtained from plants by different methods have been used for a long time[46]. In general, antibacterial activity of any EO may depend on one major compound only. However, new findings show that interactions with other compounds in the oils are also important[47].

In this study, we used two essential oils;*Citrus limon* oil and *Cinnamon cassia* oil which showed antimicrobial and antibiofilm activity against different strains of *Staphylococci*. The qualitative and quantitative chemical compositions of these EOs were determined by GC-MS.

For lemon oil, alpha-limonene (17.823%), beta Pinene (10.752%), alpha pinene (6.518%) and gamma-terpinen (6.093%) were found as major components. Many studies reported most of these major components but with different percentages. In Egypt, Abdelgaleilet al, reported that limonene (56.30%), β -pinene (8.81%), γ terpinene (6.42%), α citral (4.96%), β -citral (3.83%) and α -terpineol (3.38%) were the major components of lemon oil[48]. Also, another Egyptian study, reported limonene (56.30 %), β -pinene (8.81%) and γ-terpinene (6.425%) to be the major components [49]. In India, Paw et al, showed that the major components were limonene (55.40 %) and neral (10.39 %)[50]. Another study in Pakistan revealed that limonene (31.33 %), γ-terpinene (13.70 %) and β pinene (8.14 %) to be the major compounds[51]. A recent study in Italy, showed the six major detected compounds were limonene (53%), β-pinene (14.5%), γ-terpinene (5.9%), citral (3.8%), α-pinene (2.4%), and β-thujene (1.94%) [52]. In Tunisia, Ben Hsouna et al, reported that nine major detected components were found to be: β-Pinene (25.44%), limonene (39.74%), linalool (2.16%), α terpineol (7.30%), linalylacetate (3.01%), acétategeranyl (3.03%), nerolidol (6.91%), acetateneryl (1.74%) andfarnesol (4.28%) [53]. Also, a recent study reported that the EOs of peels of *Citrus macrocarpa* and *Citrus xamblycarpa*were found to contain D-limonene as a major compound [54]. The composition of the essential oil of lemon varies in different studies due to a variety of factors as the growing season, climate change, extraction method, geographical location and the nutritional status of the plant [55].

Our results showed that lemon oil has antibacterial and antibiofilm effects for all *Staphylococci*isolates. Many studies also reported similar findings[56]. In this study the MIC for lemon oil ranged from 1 to 0.25 % v/v. The MIC against *Staphylococci* isolatesvaried depending on the species[57]. In the study of Federman et al, who examined the effect of citrus derived oil (CDO) (Valencia orange oil) on the growth of *Staphylococcus aureus*[57]. They found that the MIC for *Staph aureus* ATCC29740 was 0.025%as determined by visual inspection. The strain used in that study was isolated from bovine mastitic milk, which may explain the relative low MIC reported in that study as the infection was acute with no biofilm producers. On the other hand, the *Staph aureus* isolates of this study were cultured from tonsillar tissue of patients with recurrent tonsillitis who were exposed to many antimicrobials and were biofilm producers.

Espina et al, found that the MIC of citrus fruit EO against *Staph aureus* was 0.5% [58]. They used *Staphylococcus aureus* (ATCC6538) strain. The Essential oil was found to cause damage to the cell well, cell membrane and mitochondrial membrane due to the fact the essential oils tend to be hydrophobic, so they disrupt the membrane, increasing membrane permeability.

In our study, the MBC was found to range from 0.5% to 2%. This disagreed with Moosavy et al, who reported that the EO of the lemon peel had MIC and MBC values of 1.25 and 5% respectively and these concentrations are higher than our results [59]. This may be explained by the difference in the bacterial strains tested as they used *S. aureus* ATCC 6538. Galgano et al, reported that *S. aureus* growth was strongly inhibited by *Citrus Lemon* (LEO), with a MIC and MBC of 5% (*v*/*v*) for clinical isolate (from dogs with recurrent cystitis) and a MIC and MBC of

1.25% (*v*/*v*) for the *S. aureus* ATCC strain 11622 [52].

Federman et al, studied the effect of the citrus-derived oil (CDO) (Valencia orange oil) on preformed *Staphylococcus aureus* biofilm, they reported a statistically significant difference in biofilm growth was observed between 0.05% CDO and the control. While, during biofilm formation, *Staph aureus* failed to form a biofilm in the presence of 0.025, 0.05, and 0.1 % CDO in broth. So, CDO was reported to have a potential preventive measure against biofilm formation[57]. Recently, Ellboudy et al showed that lemon oil possess antibacterial and antibiofilm effects against *Staph aureus*[60]. They reported a MIC, MBC and MBEC of 125, 250, and 500 μg/ ml respectively. The ability to eradicate preformed biofilm was attributed to their high proportion of phenols and aldehydes. Hydrophobicity impacts EO activity by increasing cell permeability, resulting in cell leakage[60].

Our results disagreed with Adukwu et al, who reported that lemon essential oil did not have antibacterial or antibiofilm effects against *Staphylococcus* strains [61]. They useddisc diffusion method and 0.5 %(v/v) Tween 20 for dilution of the oil instead of DMSO that was used in this study.

Regarding*CinnamonCassia* essential oil (CCEO), trans-cinnamic aldehyde 71.025%,(+)-delta-cadinene 5.241% and alpha-muurolene 4.804% were the major components in our study. Ooi et al, reported that the major components of the *Cassia* essential oil were trans-cinnamaldehyde (85%), o-methoxy-cinnamaldehyde (8.79%) and small amounts of other constituents such as benzaldehyde, alcohol, and terpenoids [62]. An analysis of purchased cinnamaldehyde by GC/MS showed the purity of this aldehyde to be high being comprised of 98% trans- and 1.27% cis-cinnamaldehyde. Both the oil and cinnamaldehyde were equally effective in inhibiting the growth of different staphylococcus strains. The important characteristic ofCCEO and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death.

Melo et al, showed that 18 substances were detected, with the major component being (E)-cinnamaldehyde, corresponding to 90.22% of the total components [63]. Netopilovaet al, reported that *Cassia* oil had 86.48% trans-cinnamaldehyde and 3.53% cinnamylacetate[47]. Another study also found that trans-cinnamaldehyde (68.52%) was found to be the major compound, followed by copaene (4.66%), benzenepropanal (3.67%), γ-cadinene (3.41%), cis-cinnamaldehyde (2.15%), α cadinol (1.85%) and cinnamyl alcohol (1.24%) [64]. In addition, Lu et al, identified 27components in the essential oil of *Cinnamomum cassia*. They reported that cinnamaldehyde (30.67%), copaene (27.71%), 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1(1-methylethyl)-(1S-cis)-naphthalene (13.55%) were the major compounds of the essential oil [65]On the contrary, our results were different from the Egyptian study of Tarek et al, who revealed that trans-caryophyllene (17.18%) was the main compound in cassia[66].

The current study reported that CCEOhad antibacterial effect against different *Staphylococci* strains with MIC ranging from 1 to 0.25 % v/v. Other studies reported different values; Melo et al, showed that the MIC of cassia oil was 0.25 % and MBC was 1%[63]. They used *S. aureus* ATCC 29213 and disc diffusion method for the determination of the MIC which may explain the lower values reported in addition to the variation in theamount of (E)-cinnamaldehyde present in the CCEO. Similarly, Firmino et al reported that CCEO MIC and MBC against*S. aureus* ATCC6538, *S. epidermidis* ATCC12228 were 0.25 and 1% v/v respectively. However, Tween 20 (2% (was used to facilitate solubilization of the oil in that study[67]. Another study reported that CCEO MIC was 0.0488 %v/v for *S. aureus* ATCC 25923 isolates [68]. The variation in the MIC values may be attributed to a difference in the chemical composition of the EO.

In agreement with the results of this study, Huang et al reported that the MIC and MBC of CCEO against *Staphylococcus aureus* ATCC 25923 were 2.5 mg/ml and 5 mg/ml respectively although they used the agar disc diffusion, and the oil was dissolved in 5%

(DMSO) [64]. They revealed the loss of the integrity of cell membranes and the vital intracellular constituents could be one of the mechanisms of action of essential oil from *Cinnamomum cassia*bark against *S. aureus*. Another study, showed that different concentration of cassia oil from 10% to 0.625 % had antimicrobial effect against *Staphylococcus aureus* and the MIC was 0.625% [69].

In this study, theMBC of CCEO ranged from 5 % to 1.250 % v/v. For the *Staphylococcus aureus*ATCC 25923 (a biofilm positive strain), it was 5% v/v. On the other hand, Melo et al, reported that MBC of CCEOin their study was only 0.25 % against *Staphylococcus aureus* ATCC 29213 (a biofilm negative strain) [63].

Regarding the biofilm eradication effect, we reported thatCCEOeradicated the existing biofilm at a concentration 5 and 10 % v/v for the clinical isolates, and 10% v/v for the *Staphylococcus aureus*ATCC 25923. However, much lower values were reported by Firmino et al,[67]. Also, a recent Egyptian study also reported that cinnamon oil extract showed potent antibacterial and antibiofilm activities against *Staphylococcus aureus* at an MBEC of 75.0 µg/mL [60].

Scanning electron microscope for*Staphylococcus aureus*ATCC 25923before treatment with EOs revealed the cellsto be spherical, smooth with an intact membrane and retained normal cell morphology but after treatment with lemon oil, at MIC 0.5% v/v there was an obvious distortionand collapsed surfacewith cells being adherent to each other showing that the bacteria were killed. Similarly, Song et al, reported the same finding using*Citrus* oil on*S. aureus*, ATCC 25923 [70]. Also, Li et al, reported the same finding using Finger citron essential oil [71].

Concerning the CCEO effect on *Staphylococcus aureus* ATCC 25923, this study revealed that it led to morphological changes like the effect of lemon oil on the *S. aureus*referencestrain. In accordance, Huang et al, reported the same effects on the *S. aureus* cells when treated with the EO of *Cinnamomum cassia* bark [64]. Many studies reported the same effect[72, 73].

In current study, scanning electron microscope for treated *Staphylococcus aureus*ATCC 25923cells with vancomycin led to similarchanges. I agreement, Singh et al,[74] and Ghaffar et al,[75]reported the same findings.

The results of this study may open the gate for future prospective research to use these oils in an edible form to achieve their effects in vivo alone or in combinations antibiotics to remove or prevent the formation of biofilms to treat chronic tonsillitis.

We conclude that biofilm producing *S aureus* are the most common cause of chronic tonsillitis, mostly sensitive to vancomycin and erythromycin. The major components of *Citrus Limon* oil were alpha-limonene, beta-I-pinene and alpha -(+)-pinene and the major components of *Cinnamon Cassia* oil were trans-cinnamic aldehyde, delta-cadinene and alpha-muurolene. *Lemon* and *Cassia* EOs had antimicrobial and antibiofilm activity at different concentrations making them hopeful potential remedies for prevention and treatment of chronic tonsillitis.

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نشرة العلوم الصيدلية
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التأثيرات المضادة للميكروبات والمضادات الحيوية للزيوت الأساسية من الحمضيات والليمون والقرفة ضد المكورات العنقودية المنتجة للأغشية الحيوية المسببة لالتهاب اللوزتين المزمن

ايلانا رافت زكي[1*] – نهلة محمد الشربيني[2] – سعاد عبد اللطيف حسن بيومي[3] – محمد مصطفي عثمان[4] – اماني محمد نافع[2] – رانيا بكري[5] – محمد سعد بداري[2]

[1] قسم الميكروبيولوجي والمناعة، كلية الصيدلة، جامعة أسيوط، مصر

[2] قسم الميكروبيولوجي الطبية والمناعة، كلية الطب، جامعة أسيوط، مصر

[3] قسم العقاقير ،كلية الصيدلة ، جامعة أسيوط، مصر

[4] قسم أمراض الأنف والأذن والحنجرة وجراحة الرأس والرقبة، كلية الطب، جامعة أسيوط، مصر

[5] قسم الباثولوجيا الإكلينيكية، معهد جنوب مصر للأورام، جامعة أسيوط ، مصر

تم إجراء الدراسة الحالية على مدى ٢٤ شهرا من مايو ٢٠٢١ إلى مايو ٢٠٢٣ على ١٦٣ مريضًا يخضعون لعملية استئصال اللوزتين بسبب التهاب اللوزتين المزمن في قسم أمراض الأنف والأذن والحنجرة وجراحة الرأس والرقبة، كلية الطب جامعة أسيوط. تم أخذ عينات من اللوزتين وزرعها على الوسائط البكتيريا التقليدية.

كان المرضى الذين شملتهم الدراسة ٩٠ أنثى (٥٥.٢%) و ٧٣ ذكرًا (٤٤.٨%) تتراوح أعمارهم بين ٣- ٢٠ عاما بمتوسط٩.٥ ±٢.٨١

تمت دراسة قدرة ٢ vitek تم تشخيص العزلات بالتقنيات البكتريولوجية التقليدية وتأكدت باستخدام نظام. العزلات على تكوين الأغشية الحيوية باستخدام طريقة لوحة الميكروتيتر وأجار الكونغو الأحمر. تم تحديد نمط الحساسية للمضادات الميكروبية للعزلات بطريقة انتشار القرص كيربي باور. تم استخلاص الزيوت العطرية من زيت الليمون الحامض والقرفة كاسيا بطريقة التقطير المائي وتم تشخيصها بطريقة كروماتوغرافيا -الغاز مطياف الكتلة تمت دراسة التأثيرات المضادة للبكتيريا والمضادة للأغشية الحيوية ٢٥٩٢٣ . S.aureus ATCC للزيوت العطرية للعزلات الاكلينيكية والسلالة المرجعية.

من بين ١٦٣ عينة من العينات الاكلينيكية، ٧٥ عينة اعطت نموا بكتيريا (٧٥/١٦٣) (٤٦.٠١٢%). تم عزل المكورات العنقودية في ٧٧.٣ (٦٤/٧٥) من الحالات. كانت المكورات العنقودية الذهبية أكثر انواع البكتيريا شيوعا بنسبة ٧٧.٣ (٥٨/٧٥) يليها المكورات العنقودية السلبية المخثرة بنسبة ٨% (٦/٧٥). باستخدام طريقة لوحة الميكروتيتر، كانت جميع العزلات منتجة للأغشية الحيوية، بينما باستخدام أجار الكونغو الأحمر، كانت ٧٨.١% فقط من العزلات منتجة للأغشية الحيوية.

أظهرت العزلات المدروسة أعلى معدلات حساسية للفانكومايسين والإريثرومايسين والتريميثوبريم/السلفاميثوكسازول ٩٠.٦%، ٨١.٣% و ٧٩.٧% على التوالي وأقل حساسية للأموكسيسيلين حمض الكلافولانيك (٢٣.٤%).

أظهر تحليل الزيوت العطرية أن المكونات الرئيسية لزيت الليمون الحامض هي ألفا ليمونين وبيتا-آي- بينين وألفا (+) بينين. وكانت المكونات الرئيسية لزيت القرفة كاسيا هي ألدهيد ترانس سيناميك ودلتا- كادينين وألفا. -مورولين.

كان لزيت الليمون الحمضي وزيت القرفة كاسيا المستخدم في الدراسة نشاط مضاد للميكروبات ومضاد للزيتين بواسطة MBIC و MIC، MBC،MBEC للأغشية الحيوية بتركيزات مختلفة. تم تحديد من ١ إلى ٠.٢٥ (حجم/حجم)، MIC طريقة التخفيف الجزئي للمرق. بالنسبة لزيت الليمون، تراوحت من ٢ إلى ٠.٢٥ (حجم/حجم)، MIC من ٢ إلى ٠.٥ (حجم/حجم) ، وتراوحت MBC وتراوحت من ٢.٥ إلى MIC من ٤ إلى ٢ (حجم/حجم). بالنسبة لزيت كاسيا تراوحت MBEC وتراوحت من ٥ MBIC من ٥ إلى ١.٢٥% حجم (حجم) ، وتراوحت ٠.٦٢٥% MBC (حجم/حجم)، وتراوحت من ١٠ إلى ٥. (حجم/حجم).MBEC إلى ٠.٦٢٥% (حجم/حجم) ، وتراوحت لعزلات مختلفة وللسلالة المرجعية ووجد أنه ٢ ميكروجرام مل (للسلالات MICتم تحديد الفانكومايسين الحساسة، العدد = ٥٨، ٤ ميكروجرام/مل للعزلات ذات الحساسية المتوسطة، العدد = (2) و ٨ S. *aureus* ATCC ميكروجرام/مل. للعزلات المقاومة، العدد = (٤). بالنسبة للسلالة المرجعية ميكروغرام/مل.MIC2 كان ٢٥٩٢٣ (MIC) لتحديد تأثير الزيتين والفانكومايسين عند قيمة ال SEM تم إجراء المجهر الإلكتروني الماسح . كانت خلايا المكورات العنقودية الذهبية ٢٥٩٢٣ . S. *aureus* ATCC و علي السلالة المرجعية الضابطة ذات شكل طبيعي، كروية، منتظمة، مع غشاء سليم محتفظ بها بشكل طبيعي. مورفولوجيا الخلية والبنية الداخلية. يكون الضرر قد تشكل على MIC بعد العلاج بزيت الليمون الحمضي أو زيت القرفة كاسيا عند قيمة سطح الخلايا مع تشويه واضح لخلايا المكورات العنقودية الذهبية، مع شكل غير واضح، ملتصق ببعضها البعض، وارتخاء جدار الخلية، وانحلال البلازما، الترشيح من محتويات الخلية، وفقدان المواد الكثيفة داخل الخلايا وفقدان المكونات الحيوية داخل الخلايا، وتلف غشاء الخلية وتمزق الغشاء. بعد ، أصبحت خلايا غير منتظمة وأصغر حجمًا ومتغيرة الحجم MIC العلاج بالفانكومايسين عند قيمة وأصبحت ملتصقة ببعضها البعض.