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Research Article

## Effect of Four Essential Oils on Cells Release Membrane and Biofilm Formation of Clinical Bacterial Isolated From Oral Infection

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### ABSTRACT

**Background:** Bacterial biofilms forming are current resistant bacterial form to the treatment of oral diseases that colonizes in the gingival and sub-gingival regions of the mouth. The present study aims to screen the anti-biofilm potential and evaluate the effect of four essential oils on cells release membrane. **Methods:** Seven type isolate bacteria obtained during previous work were screen to select those who had ability to form biofilm using Congo Red Agar method, tube method and crystal violet method. The inhibitory parameter of biofilm forming was determine using microtiter plate method. The effect of essential oil on cell membrane release of each selected bacterial was put in evidence by measuring cellular material that absorb at 260 nm and 280 nm after 0 min, 30 min and 60 min of exposure and confirm by measuring DNA, RNA and proteins release by treated cells on extracellular medium using Nanodrop 1000 spectrophotometer. **Results:** The crystal violet method shows twelve (12) strong, five (05) moderate and five (05) weak biofilm forming bacteria. The anti-biofilm activity against the oral bacteria who shown that most of essentials oils have activity on different biofilm formation and the MICs ranged from 0.31 mg/mL to 1.25 mg/mL. Concentration of intracellular material released in extracellular medium ranged from 186,56 ± 2,35 ng/μL to 766,6 ± 2,84 ng/μL for DNA, 158,06 ± 1,87 ng/μL to 628,53 ± 2,05 ng/μL for RNA and 695,9 ± 2,11ng/μL to 1125,23 ± 2,15 ng/μL for proteins. **Conclusion:** This study demonstrates that the selected EOs have a significant anti-biofilm activity, acting on the cell surface and causing the disruption of the bacterial membrane. These EOs are interesting alternative to conventional antimicrobials for the control of oral microorganisms.

**Keywords:** Anti-biofilm activity, Biofilm, oral diseases

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### INTRODUCTION

Biofilm or dental plaque is a complex microbial community composed of numerous aggregated microorganisms that attach to a surface of the teeth and become surrounded by extracellular polymeric matrix. An important characteristic of biofilm is that it is highly tolerant antimicrobial therapeutics<sup>1, 2</sup>. The unbalance of microorganism in oral biofilm results in pathological conditions such as dental caries and periodontitis<sup>1</sup>. The major features that distinguish biofilm forming bacteria from their planktonic counterparts are their surface attachment ability, high population density, extracellular polymeric substances (EPS) slime and a wide range of physical, metabolic and chemical heterogeneities<sup>3</sup>. It is now recognized that biofilm formation is an important aspect of many diseases including native valve endocarditis, osteomyelitis, dental caries, middle ear infections, ocular implant infections, and chronic lung infections in cystic fibrosis patients.

Some agents for the treatment of oral diseases are commercially available and many antibiotics are commonly used to treat oral infections such as: Penicillins and Cephalosporins, Erythromycin, Tetracycline and derivatives have been documented<sup>4</sup>. These chemicals can alter oral microbiota and have undesirable side effects such as vomiting, diarrhea and tooth staining<sup>5</sup>. Given the incidence of oral disease, increased resistance by bacteria to antibiotics, adverse effects of some antibacterial agents currently used in dentistry and financial considerations in developing countries, there is a need for alternative prevention and treatment options that are safe, effective and economical.

Essential oils (EOs) are mixtures of natural volatile organic compounds derived from the plant secondary metabolites, mainly terpenes and their oxygenated derivatives, as well as phenol-derived aromatic compounds and aliphatic hydrocarbons. EOs possess several biological activities

including antibacterial, antifungal, anti-inflammatory and antioxidant properties<sup>6,7</sup>.

In addition, essential oils and their components are gaining increasing importance because of their Generally Recognized as Safe (GRAS) status, wide acceptance by consumers, safety for the environment and less chance for pathogens to develop resistance to chemical components, due to diverse modes of mechanisms of action. Previously, we reported the chemical composition analysis *Cymbopogon citratus* DC. Staf (Poaceae), *Eugenia caryophylla* (Myrtaceae), *Mentha* sp cf *piperita* (Lamiaceae) and *Pentadiplandra brazzeana* Baill (Capparidaceae)<sup>8</sup>. Although antimicrobial efficacy of various essential oils has been reviewed previously, to the best of our knowledge, few systematic research on the antibacterial mechanism of our essential oils against a wide range of oral microorganisms has been conducted so far. Therefore, this study was undertaken in order to investigate the effectiveness of our EOs in the control of selected oral pathogens using *in vitro* models. Furthermore, antibacterial mechanism of action was investigated by measuring cellular material that absorb at 260 nm and 280 nm after some time of exposure and confirm by measuring DNA, RNA and proteins release by treated cells on extracellular medium using Nanodrop 1000 spectrophotometer. The present study aims to screen the anti-biofilm potential and evaluate the effect of four essential oils on cells release membrane.

## MATERIAL AND METHODS

### Bacterial strains used

The clinical strains used in this study are *Staphylococcus aureus*, *Streptococcus* sp, *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* isolated from the oral cavity from patients and identified during a previous study<sup>7</sup>, and *Pseudomonas aeruginosa* ATCC 27853.

### Essential oils used

The essential oil of *Cymbopogon cytratus*, *Pentadiplandra brazzeana*, *Eugenia caryophylla* and *Mentha* sp cf *Piperita* were obtained by hydrodistillation during a previous work<sup>8</sup>. Then, analyzed by Gas Chromatography with Flame Ionization Detector (GC-FID) and Gas Chromatography coupled with Mass Spectrometry (GC/SM) showed the presence of geranial (33.76 %), benzyl isothiocyanate (86.81 %), eugenol (70 %) and piperitone (50.63 %) respectively<sup>8</sup>.

### Screening of biofilm forming

During a previous study, seven bacterial species with higher frequency in oral infection were selected to detect their ability to form biofilm<sup>8</sup>. These isolates included five *Staphylococcus aureus*, one *Streptococcus* sp, three *Bacillus cereus*, two *Bacillus subtilis*, two *Enterobacter cloacae*, two *Klebsiella pneumoniae*, two *Klebsiella oxytoca* and *Pseudomonas aeruginosa* ATCC 27853. This ability to form biofilms by these strains were tested by three different technique: Congo red agar method (CRA), tube method and crystal violet method.

### Congo red agar method (CRA)

This qualitative method is based on the characteristic cultural morphology of biofilm-forming bacteria on Congo red medium. The isolates were streaked on the Muller Hinton agar (Scharlau, Spain) supplemented with 0.8 g/L of Congo red dye and incubated for 48 hours at 37° C. The production of black colonies with a dry crystalline consistency indicated biofilm formation and non-biofilm producing strains develop red colonies<sup>9,10</sup>.

### Tube method

Qualitative assessment of biofilm formation was determined by the tube staining assay<sup>11</sup>. Isolates were inoculated (100 µL) in 10ml Muller Hinton broth (MHB) supplemented with 0.5 % glucose and incubated for 24 hours at 37° C at static and rotary condition. The tubes were decanted and washed with phosphate buffer saline (PBS) (pH 7.3), dried and Stained with 0.1 % crystal violet. Excess stain was removed by washing the tubes with deionized water. Formation of biofilm was confirmed with the presence of visible film on the wall and bottom of the tube. However, the liquid interface did not indicate biofilm formation<sup>9</sup>. Biofilm formation ability was tentatively scored as strong (+++), moderate (++) , weak (+) and negative (-) by visually comparing the thickness of adherent layer.

### Crystal violet method

The ability of isolates to form biofilms in 96-well microplates was determined according to the procedure of **O'Toole and Kolter**<sup>12</sup> with modification. The isolates were grown in Muller Hinton broth (MHB) with 5 % sucrose and incubated overnight at static condition at 37° C to obtain sufficient bacterial growth. The cultures after 24 hours were diluted 100 times with the same medium and 200µL of the culture were inoculated in the 96 well plate in triplicates. The 96 well plate was incubated for 24 hours in static condition at 37° C at static condition. After respective incubation period content of each well was gently removed by slightly tapping the plates. The wells were then washed with phosphate buffer saline (PBS pH 7.3) to remove free-floating planktonic bacteria. The plates were then stained with 0.1 % (w/v) crystal violet solution. Excess stain was washed off thoroughly with 95 % ethanol and plates were kept for drying. Optical density (OD) was measured using micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of attachment to surface. The experiment was performed in triplicates and average reading was considered.

### Anti-biofilm activity

The inhibitory effect of the EOs on the biofilm biomass of the selected organisms, capable to form biofilm was investigated by the microtiter plate method. Two-fold serial dilutions of the essential oils were made and added into sterile 96 wells microtiter plates containing 50 µL of sterile Muller Hinton broth supplemented with 0.5 % glucose already inoculated with 50 µL of an overnight culture of the test organisms standardized to an optical density of 0.1 at 620 nm (equivalent to 0.5 McFarland standard turbidity). Positive control (bacterial suspension in broth) and negative control (essential oils in broth) were maintained for each concentration. Following an incubation at 37° C for 24 hours, the content of each well was gently removed by tapping the plates. The wells were washed with 200 µL of sterile distilled water to remove free floating planktonic bacteria. Biofilms formed by adherent cells in plate was stained with 0.1 % crystal violet and incubated at the room temperature for 30 minutes. Excess stain was rinsed off by thorough washing with distilled water and plates were then fixed with 200 µL of ethanol 70 %. Crystal violet contained in the bacteria was then solubilized with 70 % ethanol (Fischer, France) and the OD was read at 620 nm with a Microplate Spectrophotometer. Each test was conducted in triplicate<sup>13</sup>.

### Effect of EOs on Cell Membrane

The effect of EOs was evaluated in two steps. Firstly by detecting the presence of nucleic acids in extracellular medium after 0 min, 30 min and 60 min of exposition.

Secondly to confirm effect of EOs on cell membrane release, the concentration of DNA, RNA and proteins released by treat cells was determined using NanoDrop 1000 apparatus.

#### Detection of nucleic acids at 260 nm after different times of exposure

The leakage of cytoplasmic membrane was highlighted by determining the release of cellular materials including DNA and RNA which absorb at 260 nm into the bacterial suspensions. After following the release of biomolecules after 0 min, 30 min and 60 min, it was noticed in Figure 3 that all EOs used in this study had an effect on the cell membrane after 30 min of exposure. Damage to the membrane by EOs was marked by an increase of absorbance at 260 nm compared to the control without EOs which implies the release of biomolecules (DNA, RNA) into the extracellular medium<sup>14</sup>.

#### Quantification of release of DNA, RNA and Proteins

In previous studies, the leakage of biomolecules was determined using spectrophotometric quantification of absorbents materials at 260 nm for nucleic acids and 280 nm for proteins<sup>15, 16</sup>. In this study, the biomolecules released by bacterial isolates was determined by quantification of cellular materials released in extracellular medium, especially DNA, RNA and proteins using NanoDrop 1000 spectrophotometer (Thermo Scientific)<sup>17, 18</sup>. After an overnight bacterial culture in presence of a MIC EOs each, 100 µL of the mixture were collected, homogenized and the supernatant was measured using NanoDrop 1000 spectrophotometer. All the measurements were performed in triplicate.

#### Statistical analysis

Data were combined and analysed by analysis of variance (ANOVA). The ANOVA was performed with SPSS software (version 23.). The significant differences ( $p < 0.05$ ) were estimated by Tukey and values were expressed as mean  $\pm$  SD.

## RESULTS

#### Biofilm formation assay

The isolates were screened for the biofilm formation and were confirmed by Congo red agar (CRA) method (Table 1). Some of isolates show black colonies with a dry crystalline consistency which indicated positive for biofilm formation (Figure 1).

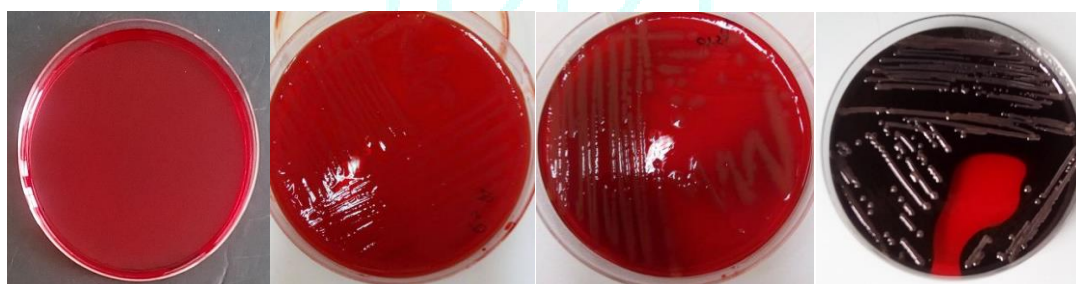
The biofilm formation was also evaluated qualitatively by tube assay (Table 1). The tubes were stained with crystal violet and some of isolates shows adherence to the walls and bottom of the test tube.

#### Micotiter plate assay

The quantitative estimation of the biofilm was done by microtiter plate assay. Optical Density (OD) was recorded at 570 nm using ELISA reader (Figure 3). The isolates were classified into: biofilm non-producers ( $OD_{570} < 0.1$ ), weakly biofilm producers ( $OD_{570} > 0.1$  to  $\leq 0.4$ ), moderate biofilm producers ( $OD_{570} > 0.4$  to  $\leq 0.8$ ) and high biofilm producers ( $OD_{570} > 0.892$ ). Among the isolate strains used, twelve (12) displayed strong ability to form biofilm showing  $OD_{570}$  range between 0.28 to 1.15 nm against a positive control. Therefore the above isolates were selected for biofilm inhibition assays.

**Table 1:** Biofilm formation by isolated strains of the oral cavity

Technique	Number of tested strain (17)		
	Strong	Modered	Absent
CRA	09	03	10
Tube method	07	09	06
Micrititer plate	12	06	04



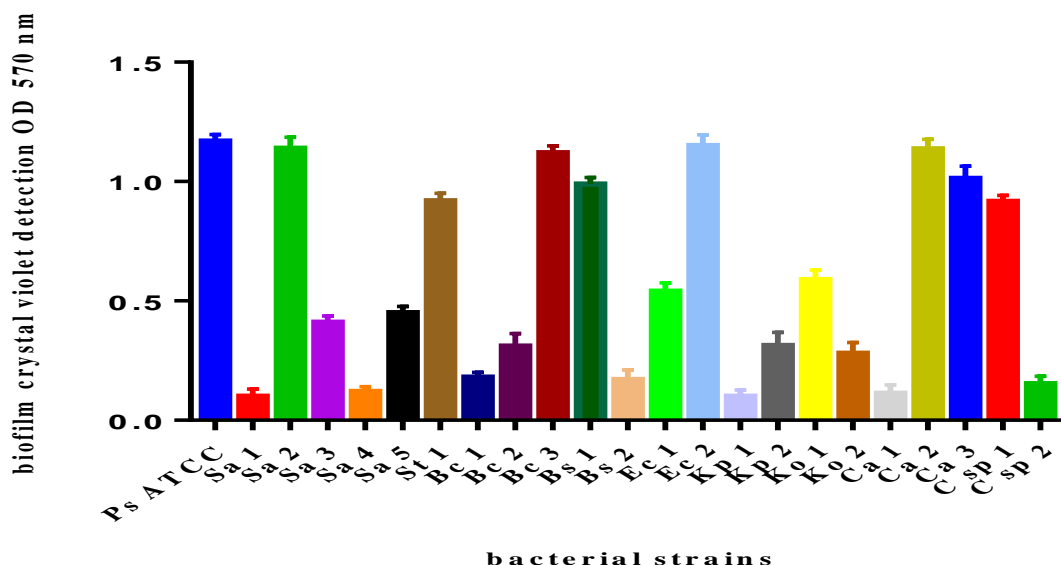
A: unseeded

B: negative slime production

C: variable

D: slime production

**Figure 1:** Slime production in the isolated strain of *Staphylococcus aureus* (Sa 2) on Congo Red Agar



**Figure 2:** Quantification by measuring OD at 570 nm of crystal violet-stained biofilms formed by oral bacterial strains in microplates for 18 h. Values are means ± SD of three independent experiments. Sa 2, Sa 3, Sa 5, St 1, Bc 3, Bs 1, Ec 1, Ec 2, Kp 1, Ca 2, Ca 3, Csp 1: high biofilm producers; Bc 1, Bc 2, Bs 2, Kp 2, Ko 2, Csp 2 : moderate biofilm producers; Sa 1, Sa 4, Kp 1, Ca 1 : biofilm non-producers.

**Biofilm inhibition assays**

The MICs of the anti-biofilm activity for all used strains are shown in Table 3. The present results demonstrate that most of essentials oils have activity on different biofilm formation and the MICs ranged from 0.31 mg/mL to 1.25 mg/mL. The EO of *Pentadiplandra brazzeana* and *Eugenia caryophylla*

were most effective against *Klepsiella pneumonia* (0.31 mg/mL) and *Bacillus cereus* (0.62mg/mL). Contrepart, the EOs of *cymbopogon citratus* and *Mentha sp cf piperita* were least efficient. We also observe that Gentamin® have activity on biofilm formation with the MICs range from 0.12 mg/mL to 0.25 mg/mL.

**Table 2:** Effect of essential oil on biofilm formation

Biofilms	Culture name	Huiles essentielles CMI (mg/mL)				Antifongique / CMI (mg/mL)	Antibiotique
		Pb	E.C	CC	Mp	Amp B	Genta
<i>Bacillus cereus</i>	Bc 3	0,62	0,62	/	/		<0.25
<i>Bacillus subtilis</i>	Bs 1	0,31	0,62	1,25	1,25		<0.25
<i>Enterobacter cloacae</i>	Ec 2	0,62	1,25	/	/		0.25
<i>Klebsiella oxytoca</i>	Ko 1	0,62	1,25	/	/		<0.25
<i>Klebsiella pneumoniae</i>	Kp 2	0,31	0,31	1,25	1,25		<0.25
<i>Staphylococcus aureus</i>	Sa 2	0,62	1,25	1,25	/		0.12
<i>Streptococcus sp</i>	St 1	0,62	0,62	1,25	1,25		0.25

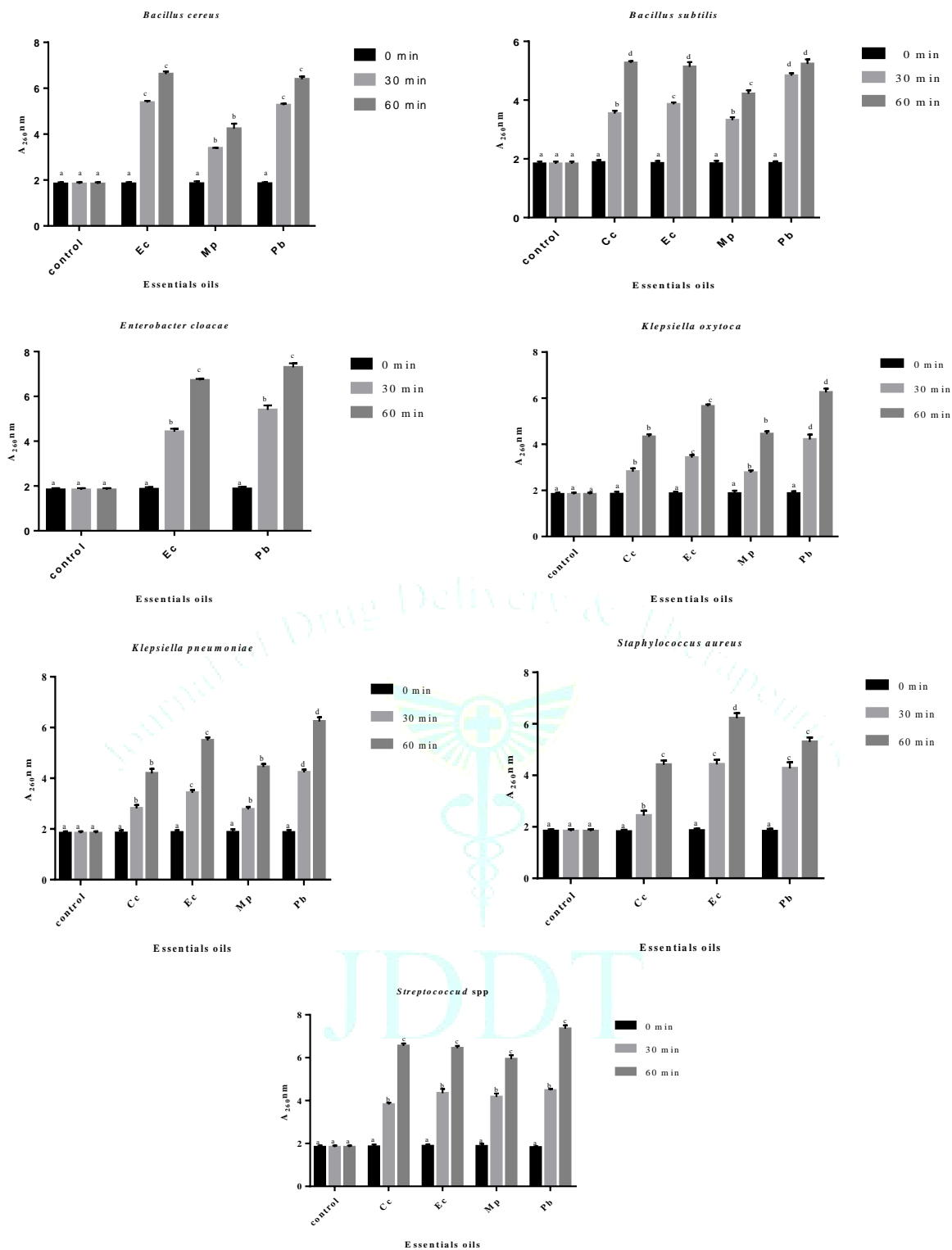
Légende : Pb : *pentadiplandra brazzeana* ; Ec : *Eugenia caryophylla* ; Cc : *Cymbopogon citratus* ; Mp : *Mentha sp cf piperita* ; Amp B : Amphotéricine B ; Genta : Gentamicine ; Id : indéterminé ; (/) : non déterminé.

**Integrity of the Cell Membrane**

**Measurement of the release of intracellular material that absorbs at 260 nm after different times of exposure**

The leakage of cytoplasmic membrane was analyzed by determining the release of cellular materials including RNA and DNA which absorbs at 260 nm into the bacterial suspensions. After following the release of biomolecules

after 0 min, 30 min and 60 min, it was noticed in Figure 4 that all EOs used in this study had an effect on the cell membrane after 30 min of exposure. Damage to the membrane by EOs was marked by an increase of absorbance at 260 nm compared to the control without EOs which implies the release of biomolecules (DNA, RNA) into the extracellular medium.



Legend: Cc: *Cymbopogon citratus*; Ec: *Eugenia caryophylla*; Mp: *Mentha sp. cf. piperita*; Pd: *Pentadiplandra brazzeana*.

**Figure 3:** Effect of *C. citratus*, *E. caryophylla*, *M. sp.cf. piperita* and *P. brazzeana* essential oils at MIC concentration on the release rate of material that absorbs at 260 nm from: *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus spp*. Data are expressed as mean values ± standard deviations (S.D.), N=3. Values with different letters are significantly different (p<0.05)

**Quantification of release of DNA, RNA and Proteins**

To confirm effect of EOs on membrane of tested bacteria, a quantification of released biomolecule was done. The results are shown in Table 2 below. It was noticed that the control made up of untreated bacteria with EOs showed no intracellular content (DNA RNA and proteins) in extracellular medium. Concentration of intracellular material

released in extracellular medium varying from 186,56±2,35 ng/μL to 766,6±2,84 ng/μL for DNA, 158,06±1,87 ng/μL to 628,53±2,05 ng/μL for RNA and 695,9±2,11ng/μL to 1125,23±2,15 ng/μL for proteins. On all the bacteria exposed to the different EOs, the EOs of *Cimbopogon citratus* and *Eugenia caryophylla* induced greater damage to the different bacteria. This is materialized by the induction of an output of a larger quantity of the intracellular content.

**Table 3:** Quantification of intracellular material in nanograms per microliter (ng/ $\mu$ L)

Microbial isolates	Biomolecules (ng/ $\mu$ L)	Essential oils				Control
		<i>C. c</i>	<i>E. c</i>	<i>M.p</i>	<i>P. b</i>	
<i>Bacillus cereus</i>	DNA	/	529,06 $\pm$ 2,41 <sup>e,f,g,h,i</sup>	353,63 $\pm$ 3,7 <sup>a,b,c,d</sup>	563 $\pm$ 7,44 <sup>g,h,i</sup>	0 $\pm$ 0
	RNA	/	431,03 $\pm$ 2,11 <sup>e,f,g,h</sup>	163,96 $\pm$ 4,99 <sup>a,b</sup>	459,16 $\pm$ 4,93 <sup>a,h</sup>	0 $\pm$ 0
	Proteins	/	1092,03 $\pm$ 3,7 <sup>d,f,g</sup>	937,30 $\pm$ 6,87 <sup>e,f</sup>	1100,36 $\pm$ 2,07 <sup>f,g</sup>	0 $\pm$ 0
<i>Basillus subtilis</i>	DNA	186,56 $\pm$ 2,35 <sup>a</sup>	357,9 $\pm$ 2,89 <sup>a,b,c,d,e</sup>	276,85 $\pm$ 3,88 <sup>a,b,c</sup>	643,82 $\pm$ 2,45 <sup>h,i,j</sup>	0 $\pm$ 0
	RNA	158,06 $\pm$ 1,87 <sup>a,b</sup>	245,9 $\pm$ 2,84 <sup>a,b,c,d</sup>	196,4 $\pm$ 3,95 <sup>a,b,c</sup>	517,67 $\pm$ 2,15 <sup>h,i</sup>	0 $\pm$ 0
	Proteins	695,9 $\pm$ 2,11 <sup>a</sup>	736,26 $\pm$ 2,25 <sup>a,b,c</sup>	703,2 $\pm$ 1,83 <sup>a,b</sup>	1101,67 $\pm$ 2,64 <sup>f,g</sup>	0 $\pm$ 0
<i>Enterobacter cloacae</i>	DNA	/	480,4 $\pm$ 4,81 <sup>d,e,f,g,h</sup>	/	575,96 $\pm$ 1,47 <sup>g,h,i</sup>	0 $\pm$ 0
	RNA	/	379,7 $\pm$ 4,12 <sup>d,e,f,g,h</sup>	/	448,3 $\pm$ 1,73 <sup>f,g,h</sup>	0 $\pm$ 0
	Proteins	/	1063,56 $\pm$ 1,6 <sup>e,f,g</sup>	/	1121,6 $\pm$ 2,96 <sup>f,g</sup>	0 $\pm$ 0
<i>Klebsiella oxytoca</i>	DNA	290,8 $\pm$ 2,48 <sup>a,b,c</sup>	358 $\pm$ 2,16 <sup>a,b,c,d,e</sup>	302,53 $\pm$ 2,57 <sup>a,b,c</sup>	435,76 $\pm$ 2,49 <sup>c,d,e,f,g</sup>	0 $\pm$ 0
	RNA	222,43 $\pm$ 3,06 <sup>a,b,c</sup>	238,30 $\pm$ 1,65 <sup>a,b,c,d</sup>	285,40 $\pm$ 1,75 <sup>b,c,d,e</sup>	298,33 $\pm$ 4,68 <sup>b,c,d,e,f</sup>	0 $\pm$ 0
	Proteins	948,03 $\pm$ 4,75 <sup>d,e,f</sup>	785,6 $\pm$ 5,36 <sup>a,b,c,d</sup>	704,6 $\pm$ 2,35 <sup>a,b</sup>	1046,10 $\pm$ 3,21 <sup>e,f,g</sup>	0 $\pm$ 0
<i>Klebsiella pneumoniae</i>	DNA	671,16 $\pm$ 2,02 <sup>i,j</sup>	766,6 $\pm$ 2,84 <sup>j</sup>	314,13 $\pm$ 3,99 <sup>a,b,c,d</sup>	532,76 $\pm$ 2,3 <sup>f,g,h,i</sup>	0 $\pm$ 0
	RNA	424,23 $\pm$ 1,7 <sup>e,f,g,h</sup>	628,53 $\pm$ 2,05 <sup>i</sup>	292,76 $\pm$ 6,4 <sup>b,c,d,e</sup>	482,96 $\pm$ 2,75 <sup>g,h,i</sup>	0 $\pm$ 0
	Proteins	1125,23 $\pm$ 2,15 <sup>f,g</sup>	1234,33 $\pm$ 2,97 <sup>g</sup>	735,73 $\pm$ 2,45 <sup>a,b,c</sup>	701,86 $\pm$ 1,51 <sup>a,b</sup>	0 $\pm$ 0
<i>Staphylococcus aureus</i>	DNA	241 $\pm$ 2,15 <sup>a,b</sup>	189,40 $\pm$ 3 <sup>a</sup>	/	369,86 $\pm$ 1,62 <sup>b,c,d,e,f</sup>	0 $\pm$ 0
	RNA	186,63 $\pm$ 1,71 <sup>a,b</sup>	303,33 $\pm$ 2,35 <sup>b,c,d,e,f</sup>	/	291,33 $\pm$ 2,51 <sup>b,c,d,e</sup>	0 $\pm$ 0
	Proteins	876,9 $\pm$ 2,13 <sup>a,b,c,d,e</sup>	785,03 $\pm$ 2,51 <sup>a,b,c,d</sup>	/	825,43 $\pm$ 1,68 <sup>a,b,c,d</sup>	0 $\pm$ 0
<i>Streptococcus spp.</i>	DNA	566,06 $\pm$ 2,89 <sup>g,h,i</sup>	435,53 $\pm$ 3,15 <sup>c,d,e,f,g</sup>	482,63 $\pm$ 3,30 <sup>d,e,f,g,h</sup>	195,36 $\pm$ 2,11 <sup>a,b</sup>	0 $\pm$ 0
	RNA	345,56 $\pm$ 3,4 <sup>c,d,e,f,g</sup>	239,83 $\pm$ 3,4 <sup>a,b,c,d</sup>	387,23 $\pm$ 3,33 <sup>d,e,f,g,h</sup>	126,76 $\pm$ 1,74 <sup>a</sup>	0 $\pm$ 0
	Proteins	894,16 $\pm$ 1,47 <sup>c,d,e</sup>	899,80 $\pm$ 1,6 <sup>c,d,e</sup>	1060,26 $\pm$ 1,7 <sup>e,f,g</sup>	888,3 $\pm$ 1,77 <sup>b,c,d,e</sup>	0 $\pm$ 0

Legend: ND: Not Determined; *P. b*: *Pentadiplandra brazzeana*; *M. p*: *Mentha sp cf piperita*; *E. c*: *Eugenia caryophylla*; *C. c*: *Cymbopogon citratus*. (/): not determined. Data are expressed as mean values  $\pm$  standard deviations (S.D.), N=3. Values with different letters are significantly different (p<0.05)

## DISCUSSION

Researchers have investigated the strategies employed by microorganisms to produce biofilms and to understand the pathogenesis. They discovered that biofilm producing bacteria secrete certain chemicals that protect them from disinfectants and antimicrobials and phagocytic host immune systems<sup>19</sup>. Several conventional methods of detecting biofilm production have been established, such as the standard Tube Method<sup>11</sup>, plate method<sup>20</sup>. Using this different method for testing biofilms production have been reported by other authors<sup>9</sup>. In this study, 17 strains of isolated oral bacterial were used to demonstrate biofilm forming ability.

Among the strains revealed strong biofilm formation using spectroscopic method. Biofilm formation among these strains has been reported by several other workers<sup>21, 24, 23</sup>.

Antibiofilm activity of EOs against oral strains may kill planktonic bacteria shed from the biofilm surface; however, they fail to eradicate those embedded within the biofilm, which can then subsequently act as a source for recurrent oral infection. At present, conventional systemic therapies, using standard antimicrobial agents, represent the main strategy for the treatment and prevention of biofilm infection. Therefore, EOs namely *Pentadiplandra brazzeana*, *Eugenia caryophylla*, *Cymbopogon citratus* and *Mentha sp cf piperita* were tested for their antibiofilm activity against the test strains. In our study, antibacterial susceptibility studies

of the test strains was demonstrated<sup>8</sup>. The present results demonstrate that the concentration of EOs required to inhibit the biofilm form was on average 2 times higher than the MIC of the planktonic form<sup>8</sup>. Similar trend was observed by several other workers<sup>24, 25</sup>. The reasons for the resistance of cells embedded in biofilms may include limited diffusion of EOs into the biofilm or decreased bacterial growth. Some EOs can react with the biofilm matrix and, on the other hand, the cells in biofilms can adapt and form protected phenotypes<sup>26</sup>.

Regardless of how promising the results are, additional studies are necessary to assess the cytotoxicity of the oil against epithelial cells and keratinocytes, and to determine the antimicrobial effects of our used EOs oil in combination with the oils of other plants, substances, or preservatives contained in mouthwashes used *in vivo*, to evaluate its antimicrobial potential.

The bacterial membrane serves as a structural component which may become compromised during a biocidal challenge such as exposure to anionic, cationic or neutral biocides. Therefore, release of intracellular components is a good indicator of membrane integrity. Loss of significant 260-nm-absorbing materials with increase of time interval, suggesting that nucleic acids and some protein were lost through a damaged cytoplasmic membrane. Similar studies were carried out by<sup>27</sup>.

The effects of EOs may be due to their components as well as a synergy between the different antimicrobial molecules contained in these EOs. Several monoterpenes were found to affect the structural and functional properties of the lipid fraction of the plasma membrane of bacteria, causing intracellular materials to leak<sup>28</sup>. Release of intracellular components is a good indicator of membrane integrity. Small ions such as potassium and phosphate tend to leach out first, followed by large molecules such as DNA, RNA, and other materials, when treated with a suitable antimicrobial. Since these nucleotides have strong UV absorption at 260 nm, they are described as '260 nm absorbing materials' and this method is widely used in determining membrane integrity parameters<sup>29,30</sup>.

## CONCLUSION

At the end of this study whose general objective was to evaluate antibacterial mode of action and to screen potential anti biofilm agents. The results of this study revealed that, many microorganisms present in the mouth like *Bacillus cereus*, *Klebsiella pneumoniae*, *Streptococcus sp* and *Staphylococcus aureus* have ability to form biofilm. Selected Eos were found to have a potential inhibitory effect on oral bacteria. This effect of EOs was associated with their ability to disrupt the bacterial membrane, causing loss of membrane integrity and leakage of 260 nm absorbing material. EOs were found to have anti-biofilm activity against the isolated germ, and may be suggested as a new potential source of natural antimicrobial for the prevention and control of bacterial infections of oral cavity.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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