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Assessment the Effect of Some Reagents on the Planktonic Cells and Biofilms of Red Complex Periodontal Pathogens

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Abstract

The current study aimed to investigate the effectiveness of four regents; two naturals, olibanum and alum, and two standards, ciprofloxacin (CIP) and chlorhexidine (CHX) to affect the growth and biofilm of three types of periodontal pathogens, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, "the red complex group". Clinical isolates of the red complex pathogens were isolated from chronic periodontitis. They were identified by phenotypic properties and molecular method. The inhibitory activity of the four reagents was tested by microdilution method. Minimal inhibitory concentration (MIC) on the bacterial plankton and minimal biofilm inhibitory concentration (MBIC) on biofilm of the four reagents in a single and combinational use was determined on mono- and polymicrobial populations. Simple linear regression modeling was used to explore the effect of each reagent and determine MICs and MBICs. All reagents showed inhibition activity against the growth of mono- and polymicrobial planktonic population. MIC values on polymicrobial growth were higher than on monomicrobial growth and MBICs were much higher. All reagents had antibacterial activity on a monomicrobial biofilm with greater significant effect on *T. denticola* then *T. forsythia* and *P. gingivalis*. On polymicrobial biofilm, just olibanum continued showing its effect whilst CHX was less effect and both alum and CIP had no effect. Combinational use with Olibanum encouraged the effect of other regents on polymicrobial biofilm. This combination is a promising medicated preparation to combat the subgingival plaque of red complex pathogens.

Keywords: Oral biofilm; resistance of periodontal pathogens; treatment of periodontitis.

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1. Introduction

Periodontitis is one of the major public health problems in all societies. It is a polymicrobial infection induced by anaerobic bacteria resident together in subgingival biofilms [1]. Subgingival biofilm involves large numbers of different species embedded in an extracellular polymeric substance (EPS) within which cells are in limited growth and metabolic activities and higher frequency of mutations; these facts make bacteria more resistant to antibacterials and lead to recalcitrant infections [2, 3]. Practically, it is difficult to selectively target only the actual pathogens among the hundreds different species present in periodontal plaque [4]. In most cases scaling and root planning (SRP) must be followed by chemotherapy prescriptions for better improving the healing [5]. Unfortunately, prolong use of such antimicrobials may lead to disagreeable side effects and development of microbial resistance which is considered a major threat in medicine and public health [6]. In addition to, differences in the effect of antimicrobials among oral bacteria and between biofilm and plankton cells, less activity of the common antimicrobials in the treatment of anaerobic bacterial infections and multidrug resistant species along with the development of several resistance mechanisms were recorded [7,8]. *P. gingivalis*, *T. forsythia* and *T. denticola* "the members of red complex group" were found to be the most frequent responsible pathogens [9] which invade deeper into pocket epithelium to persist with other pathogens a polymicrobial biofilm far away to be reached by SRP and more resist to antimicrobials [10]. In the same context, Rams and his team (2020) [11] recorded resistance of several periodontal pathogens to several antibiotics commonly used in the course of periodontal treatment. These indications drew attention to look for newer antimicrobial agents and highlight the importance for screening of new oral formulations with more predictive clinical activity. As safe substituents, wide range of different natural materials is now under survey for medical benefits and infections control [12]. Among these natural materials, the current study planned to investigate the efficiency spectra of the natural materials, olibanum and alum versus two standard antibacterials, ciprofloxacin (CIP) and chlorhexidine (CHX) on the planktons and biofilms of the three types of red complex periodontal pathogens.

2. Materials and Methods

2.1 Bacterial isolation and identification

Clinical isolates of the three types of red complex pathogens were isolated from chronic periodontitis by the aid of specialized dentists and transported in phosphate buffered saline (pH=7.2) to the Microbiology Lab in College of Sciences/ University of Mosul**.** Samples were streaked into three types of media prepared for the three types of bacteria, Schaedler Anaerobe Agar for *P. gingivalis*, *Tannerella forsythia* (TF) agar for *T. forsythia*, and Trypton Yeast extracts Gelatin Volatile fatty acids and Serum (TYGVS) agar for *T. denticola*. The plates were incubated four days in anaerobic Jar using microaerophilic generation system, CampyGen (Oxiod Ltd, Japan) at 37°C. The isolates of the three types of red complex group were identified by phenotypic characters depending on Moll, (2016) [13] and also by the molecular method, loop mediated isothermal amplification technique.

2.2 Natural material and standard antibacterial solutions

Olibanum (*Boswellia sp.*) and alum (potassium alum) were purchased from local perfumer market in Mosul City. Standard drugs, ciprofloxacin (CIPLA LTD, India) and chlorhexidine digluconate (Scitra Co., UAE) were purchased from local pharmacy. Stock solutions of 0.5g/ml olibanum, 0.6g/ml alum, 120 mg/ 100 ml CHX and 200 mg/ 100 ml CIP were two- fold diluted and tested for their antibacterial effect on mono- and poly- microbial cells, biofilm formation and mature biofilm.

2.3 Effect of the four agents on the mono- and poly-microbial plankton and biofilm formation

The test was done by microdilution method in 96-flate bottom well plate. Procedure of Ong and his colleagues (2017) [14] was depended as follows: three days aged inoculum of each bacterial type was used to prepare a suspension in its correspond medium (free of antibiotics) equal to McFarland 0.5. In a 96- flat well microdilution plate, 100 µl of different concentrations of the agents were added either alone or in combination with olibanum. 100 µl of one bacterial type was added to the wells as monomicrobial populations. In another wells 50 µl of two bacterial types or 33 µl of the tree bacterial type were added as polymicrobial populations. Wells contained only bacterial inoculum with no antibacterial agent or contained bacteria- free medium were set as positive and negative controls respectively. The plates were sealed and incubated anaerobically for 72 hr. Plankton's growth was measured as an absorbance reads using a microplate reader at 630nm. For biofilms measuring, the contents of the wells were decanted and the wells were washed several times with distilled water to takeout the non-adherent cells. Attached biofilms were stained with 100 µl of 0.1% crystal violet for 15 min., and then washed several times with distilled water. The stained biofilms were extracted with (80% ethanol + 20% acetone) and after 15 min. the absorbance at 630nm was read using a microplate reader. All readings were plotted versus the concentration of reagents in a linear regression modeling to explore the effect of reagents' concentrations on the bacterial plankton and biofilm formation and determine the minimal inhibitory concentration (MIC) and minimal biofilm inhibitory concentrations (MBIC).

2.4 Effect of the four reagents on mono- and polymicrobial mature biofilms

For each bacterial type, a suspension in antibiotic free- medium equal to McFarland 0.5 was made. 200 µl of the suspensions was added to the well plates either as a mono- or polymicrobial inoculum as described above and incubated without the addition of antibacterial agents. After 3 days of anaerobic growth, the contents of the wells were decanted and the wells were washed. Fresh medium was added and also 100 µl of different concentrations of the reagents either alone or in combination with olibanum. The plates were re- incubated for 24 hr. Then, the contents of the wells were decanted and the wells were washed several times. The biofilms were stained and extracted as described above and their absorbance readings at 630nm were plotted in a linear regression modeling as written earlier.

2.5 Statistical analysis

Statistical significant for the effect of each reagent in a single and combinational use within bacterial plankton or biofilm type was analyzed by one sample *t*- test. Comparing the effect of each reagent between all bacterial groups, and all reagents on a bacterial group, and between single and combinational use were tested by ANOVA

test. The significant effects were considered at p value ≤ 0.05.

3. Results

Bacterial isolates that were black hemolytic on Schaedler blood agar and gram negative coccobacilli cells were selected to be *P.gingivalis*. Tiny white non hemolytic colonies on TF agar and gram negative bacilli cells were chosen to be *T. forsythia*. *T. denticola* isolates were pointed as white- hazy colonies on TYGVS agar with spiral forms cells and negative to gram stain. These isolates were also diagnosed with the molecular LAMP method in which the isolate that changed the color of reaction mixture from pink to yellow at 65°C within 30 min was assigned to be the desired species.

3.1 Effect of the four reagents on the mono- and polymicrobial planktons, biofilms formation and mature biofilms of red complex group

The antibacterial effect of each reagent was determined by the negative correlation between the reagent's concentrations and the absorbance which was evident by the negative value of the regression coefficient in the simple linear regression equation. MICs and MBICs were the lowest concentrations giving the negative correlation. Statistical significant was considered at p value ≤ 0.05. The concentrations that were in positive correlation with the growth and showed statistical p value > 0.05 were determined as ineffective.

3.2 The results on mono- and polymicrobial planktonic populations

All of the four reagents affected the growth of planktonic populations as it was proved by the negative correlation and statistical *p*- value lower than 0.05. On monomicrobial populations of the three types of red complex pathogens, olibanum had MICs of (1.5- 1.8) mg/ml, alum (1.7- 2.3) mg/ml, CHX (0.037- 0.04) mg/ml, and 0.0039 mg/ml of CIP. For each regent, there was no significant difference of the effect between bacterial groups. When the population involved two or three types, the reagents still exerted their effect by giving negative correlation with the growth as it is clear in Figure 1; however, they required higher MICs to show negative correlation and *p*- value less than 0.05 against the polymicrobial population involved the three types. Olibanum recorded of MIC 2.4 mg/ ml, alum 3mg/ml, CHX 0.05 mg/ml and CIP 0.005 mg/ ml as listed in Table 1. Combination with olibanum had strengthen the effect of all reagents on polymicrobial plankton by lowering their MICs till 0.4 mg/ ml of olibanum and alum, 0.006 mg/ ml CHX, and 0.0004 mg/ ml CIP, as are listed in Table 1; and also increasing the statistical meaning of the effect. ANOVA test showed that the most statistically significant was olibanum with CIP, then CHX and alum.

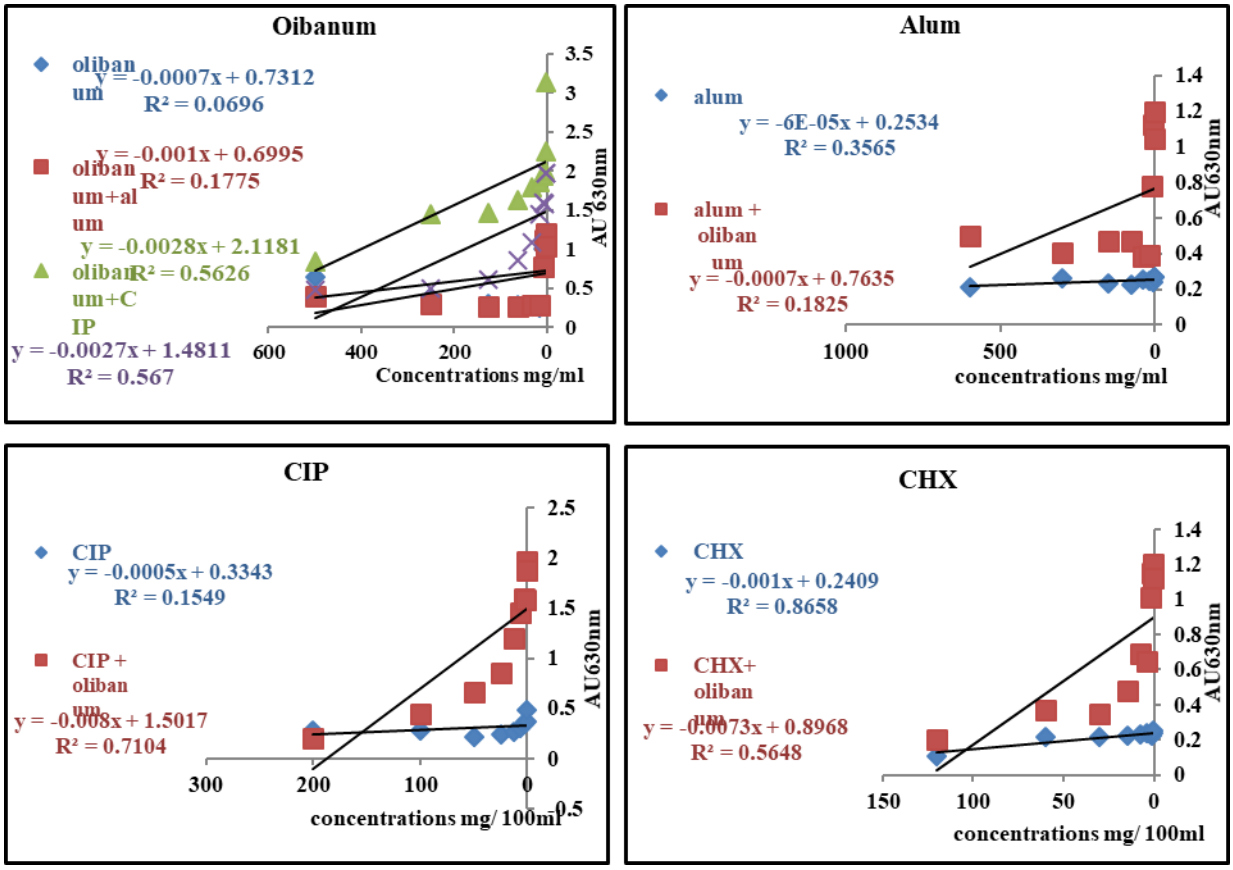

Figure 1: Linear regression modeling of the antibacterial effect on the polymicrobial plankton

Table 1: MIC values of the antibacterials in a single and combinational uses for inhibiting the growth of polymicrobial plankton

Reagents	MIC (mg/ml)	
	single use	combinational use
Olibanum	2.4	0.4
Alum	3	0.4
CIP	0.005	0.0004
CHX	0.05	0.006

3.3 The results on the formation of mono- and polymicrobial biofilm

The four reagents were able to interfere with the formation of monotypic biofilm of the three types of red complex pathogens by showing negative linear correlation and *p*- value < 0.05 by one sample *t*- test. Their MBICs were 4-5 mg/ ml olibanum, 7.7- 9.3 mg/ml alum, 0.25- 0.3 mg/ ml CHX, and 0.013- 0.015 mg/ml CIP.

ANOVA comparison showed that the statistical meaning of the effects was greater on the monotypic biofilm of *T. denticola*, then *T. forsythia* and later *P. gingivalis*. It was also showed that olibanum had the superior statistical significant than the others. Participation of two or three bacterial types in the biofilm required higher MBICs of olibanum at 11.5 mg/ ml and CHX at 0.6 mg/ml for persisting activity on a polymicrobial biofilm of red complex pathogens although with significant meaning less than that on monotypic biofilm. Alum at 12 mg/ ml and CIP at 0.02 mg/ ml were able to interfere with polymicrobial biofilm involved *T. forsythia* with *T. denticola* or with *P. gingivalis*, but unable to affect biofilms if *P. gingivalis* with *T. denticola* were the participants as they showed positive correlation in linear regression modeling and *p* value >0.05. Figure 2 clarifies the effect of all regents on polymicrobial biofilm of red complex pathogens. Combinational use fortified the effect of olibanum and CHX on a polymicrobial biofilm of red complex pathogens by lowering their MBICs to 1.2 and 0.062 mg/ ml respectively and also, rendering alum and CIP to be in negative correlation with statistical meaning at *p*- value ≤ 0.05 till 3 and 0.0012 mg/ ml MBIC respectively. These data are evident in figure 2. According to ANOVA test, olibanum with CHX was statistically the most significant then with CIP and alum, and the effect of each reagent in combination proceed significantly greater than the effect on monotypic biofilm in a single use.

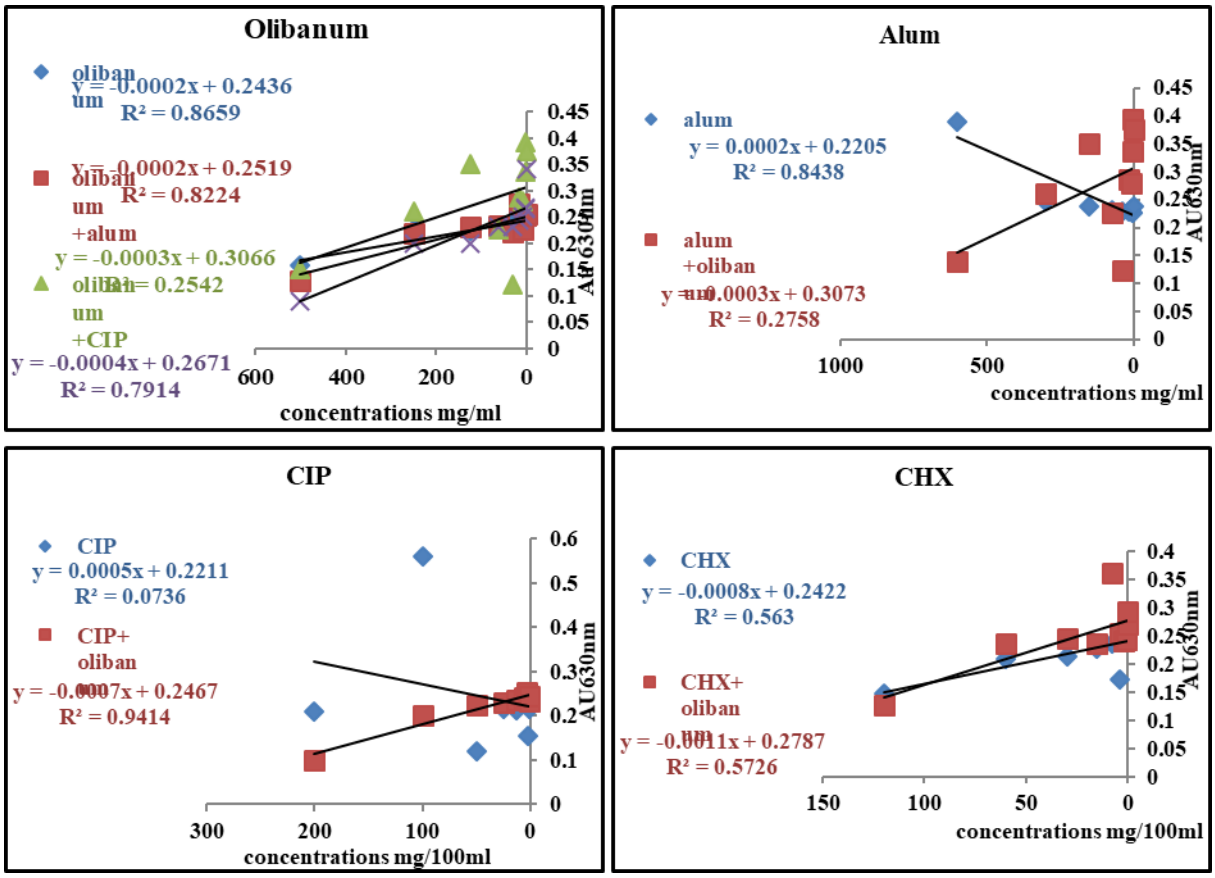

Figure 2: Linear regression modeling for the antibacterial effect on the formation of polymicrobial biofilm

3.4 The results on mono- and polymicrobial mature biofilms

All reagents showed significant activity at *p*- value ≤ 0.05 on bacteria embedded in a mature monotypic biofilm

of each type of red complex pathogens after 24 hrs. exposure. MBIC values were 7.5- 10.2 mg/ ml olibanum, 15.5- 18.7 mg/ml alum, 0.3- 0.4 mg/ ml CHX, and 0.031- 0.041 mg/ ml CIP. The negative correlation of all reagents had greater statistical meaning on monotypic biofilm of *T. denticola*, then *T. forsythia*, and later on *P. gingivalis*. ANOVA test proved the superior significant of olibanum, followed by CHX, alum, and then CIP. On the polytypic mature biofilm, just olibanum showed the profit adverse effect with MBIC on biofilm involve the three types of red complex pathogens at 31 mg/ ml, although with statistical significance less than that on monotypic biofilm. CHX at 1.2 mg/ ml (equal to the commercially available, 0.12%) showed negative correlation with significant *p*- value on polymicrobial biofilm involved just two bacterial types; but no effect on polymicrobial biofilm involved the three types of red complex pathogens. MBIC values of 37.5 mg/ ml alum and 0.05 mg/ ml CIP showed negative correlation on polymicrobial biofilm involved *T. forsythia* and *T. denticola* with significant *p*- value. If *P.gingivalis* participated, both reagents had no effect as they gave a positive correlation with the biofilm absorbance and *p*- value >0.05. These results on polymicrobial biofilm of red complex pathogens are represented in Figure 3 which also reflects the beneficial of combinational use to fortify the effect of olibanum to be active at MBIC 5 mg/ ml and strengthen other regents to exert adverse effect at MBICs 0.12, 6, and 0.002 mg/ml of CHX, alum, and CIP respectively. The greater statistical meaning determined by ANOVA test was olibanum with CHX, then alum, and CIP and the effect of each reagent in combination continued significantly greater than the effect on monotypic biofilm in a single use.

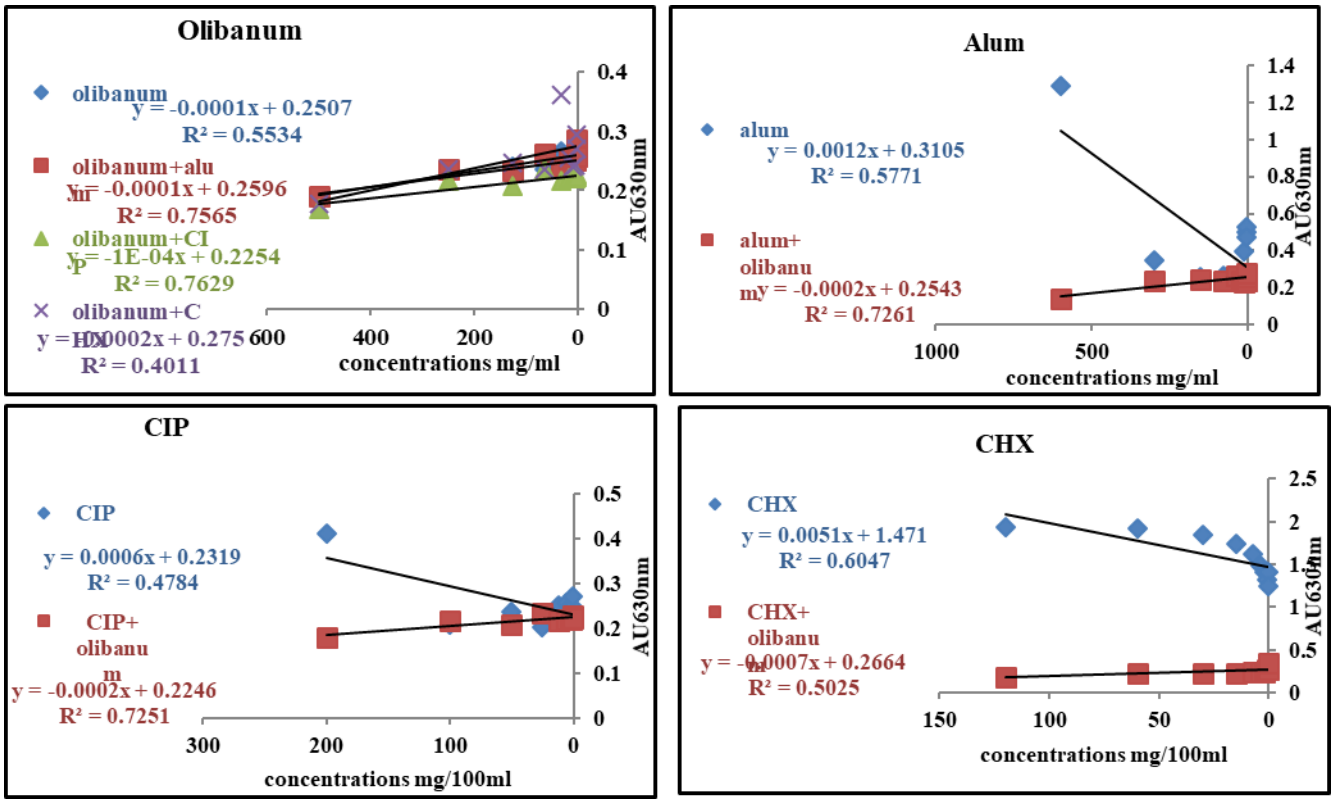

Figure 3: Linear regression modeling of the antibacterial effect on the mature polymicrobial biofilm

4. Discussion

Because periodontitis is a polymicrobial infection caused by the cooperative pathogenicity between several bacterial species, one of which, the red complex group that co-present in a biofilm life style [1]; so the current study tested the validity of the antibacterial reagents against plankton, biofilm formation and mature biofilm of bacterial types present in a single type or mixed populations (two or three types). The action of the antibacterials was tested in a single and combinational use with olibanum depending on the results of our pervious study which revealed that combining with olibanum was the greater significant than the combination between other reagents [15]. All reagents showed effectiveness on the cells in mono- and polymicrobial planktons. Their effect was lowered on biofilms especially the polymicrobial biofilms. The effect of all reagents was significantly greater on the monotypic biofilm of *T. denticola* while biofilm of *P. gingivalis* was less affected. *P. gingivalis* was known to be the key stone pathogen that adheres tightly and produces larger amount of biofilm in subgingival tissues or microtitter plates to promote the adhesion of *T. forsythia* and *T. denticola* which are less producer and poor colonizer [16]. This explains the reason of the difference in the effect between the types of biofilm. The synergistic cooperation between these three types to form excessive amount of tightly bounded polymicrobial biofilm was also proved [17] which elucidates the less activity of antibacterials on such biofilm. It was also demonstrated that communication between these types through quorum sensing by gene expression including genes of antibiotics resistance and syntrophic mutualism encourage their synergistic production of polymicrobial biofilm which were considered the most prominent phenomena in the pathogenicity of periodontitis [2]. In the same context, other scientists demonstrated that cells within biofilm are of inherent antibiotics resistance, diminished metabolic activities and embedded in a barrier of EPS, hence, are being more protected from the antibacterial effects compared with their planktons [3, 18]. The cation compound, CHX acts by distortion the integrity of cell envelops [5]. It affected all cells in the planktons or biofilms although its activity was reduced on mature polymicrobial biofilm. CIP acts on multiplying cells by targeting the enzymes DNA gyrase and topoisomerase thus disrupting cells' duplication [4]. It affected all multiplying cells in the planktons, but the activity lowered on biofilms because cells in the biofilm are with lowered metabolic and replication activities. By the acidic nature of its solution which is unfavorable for bacterial growth and proteins and enzymes functions [19], alum acted on planktons and biofilms as flocculating agent, but however, its activity was loosen on polymicrobial biofilms. By its multifunctional constituents of boswellic acids, phenolic acids, oxidizing and digestive enzymes, and the sticky nature of gum resins which affect cell's wall, membrane, and multiplication [20, 21, 22], olibanum was able to affect all bacterial groups either their planktons, biofilms formation and proceed to fight mature biofilms. In combination, the action of these reagents will cooperate to exert stronger action on the different lifestyles of polymicrobial populations. In most clinical cases, the commercial drugs are prescribed without isolating the causative agent and performing the sensitivity for these prescriptions. In addition to, it is of important consideration when studying infections that caused by mixed bacterial types, as in periodontitis, to isolate the almost suspected species and perform susceptibility tests of polymicrobial mix to mimic their occurrence in the host tissues and exactly determine the most benefit antibacterials; because the activity will be reduced on the polymicrobial existence and more particularly on the biofilm. Therefore, the current study tested the activity of antibacterials against the polymicrobial planktons, biofilms formation and mature biofilms to mimic the *in vivo* pathologic processes occurred during periodontal

infections and plaque formation. There is also a necessary demand for periodically performance of susceptibility tests of the popular antibacterials to demonstrate the continuing activity and determine the effective concentrations and period of persistence in human body fluids as it evident in the current study that the effective concentrations of CIP were higher than its level in gingival crevicular fluid (GCF) which was determined to be 0.00338- 0.00124 mg/ ml after 2- 7 hr. respectively after 500 mg dose [23]. The current study found that the effect of antibacterials was lower in state of polymicrobial presence compared to the monomicrobial growth especially in the biofilms. For instance, in the single use, CIP and alum had MICs on polymicrobial plankton at 0.005 and 3 mg/ml respectively. They affected the formation of polymicrobial biofilms involved two bacterial types at MBIC of 0.02 and 12 mg/ ml respectively (4X MIC) with no effect on biofilm involved the three types of bacteria. On mature biofilms, they affected polymicrobial biofilm of *T. forsythia* with *T. denticola* at 0.05 and 37.5 mg/ ml respectively (10- 12X MIC) with no effect of other types of polymicrobial biofilms. Re- mentioned that, the mean levels of CIP in human GCF are lower than the effective concentrations recorded by the current study. CHX had MIC on polymicrobial plankton at 0.05 mg/ ml, MBIC on the formation of polymicrobial biofilm at 0.6 mg/ ml (12X MIC) and 1.2 mg/ ml (24X MIC) on mature biofilms of two bacterial type with no effect on biofilm involve three bacterial types. Olibanum just persisted its activity on the polymicrobial presence involved the three types of bacteria with MIC 2.4 mg/ml on plankton, MBIC 11.5 mg/ml (~ 5X MIC) to interfere with biofilm formation and 31 mg/ml (~ 13X MIC) on mature biofilm. In accordance to our findings, several previous authors [4, 5, 14, 24] examined the validity of the most commonly used therapeutics in treatment of periodontal infections, the locally 0.12% w/v CHX and cetylpyridinum, and systematically CIP, Amoxicillin (AML) and azithromycin (AZT), they concluded the development of bacterial resistance and diminished anti- action by 10 times on polymicrobial mix particularly the biofilm after recording MIC and MBIC of the antibiotics greater than their levels in GCF. Their recommendations were the combinational use of these antibiotics with 250mg metronidazole (MTZ) to reach the effective concentrations; they also recorded that combination of CIP with MTZ was more inhibitor than AML with MTZ. Other previous reports recorded the superiority of 0.2% w/v CHX (200 mg/ 100 ml) upon nine tested mouth rinses against plankton, but they needed 10X concentrations to withstand monomicrobial biofilm and 10- 100X concentrations for multispecies biofilm [25, 26]. One of the *in vitro* tested proposals to improve the action of CHX on mono- species mature biofilm of *Strep. mutans*, and *S aureus* were the co-use with direct current at 28µA/cm^2 [3] or 5882µA/ cm^2 against the mature biofilm of *P. gingivalis* [27]. Others referred to the possible dependence on plant-derived natural compounds as an alternative source for novel therapeutics which can encourage or renew the activity of standard drugs. Furiga and his team, (2013) [28] submitted the use of grape seed extract (GSE) to improve the action of the commercial mouth rinses on the microbial cells and biofilms. In addition to their potential antibacterial features, many reports have definite some natural compounds as efflux pump inhibitors or resistance-modifying agents via enhancing the activity of a specific antibiotic, these benefits permitted their classification as modifiers of antibiotic activity [29, 30]. In the same context, our study suggested the beneficial combination with olibanum to resolve the invalidity of other reagents as it improved their action on the polymicrobial populations involved the three types of pathogens by lowering the MICs and MBICs values and increasing the statistical significance of their effects and endowing the inactive reagents potentials to show effect on biofilm involved the three bacterial types. For instance, in combination, olibanum interfered with the formation of polymicrobial biofilm at MBIC 1.2 mg/ ml (less than MIC of the single use) and 5 mg/ ml on mature biofilm (2X MIC on

plankton of the single use). CHX became effective at MBIC 0.062 mg/ ml (1.2 X MIC) on the formation of polymicrobial biofilm and 0.12 mg/ ml (2.4 X MIC) on mature biofilm. CIP had rendered effective at MBIC 0.0012 mg/ ml against the formation of polymicrobial biofilm and on mature biofilm at 0.002 mg/ ml, both are less than the antibiotic's MIC on plankton in a single use and at the mean level of the antibiotic in human GCF. Alum became active at MBIC 3 mg/ ml to affect polymicrobial biofilm formation and at 6 mg/ ml (2X MIC) to affect mature polymicrobial biofilm. Our findings declared that instead of using higher concentrations of CHX or CIP, incorporation of olibanum in combination with alum "as pure natural preparations" or with CHX or CIP are of significant medical values in the course of treating persisting periodontal plaque. The current study describes olibanum as one of a new anti- subgingival plaque natural substance by its own anti-action and also its ability to renovate the activity of other antibacterials. The application of olibanum can also compensate the combination of CIP with MET and the need to increase the antibiotic dose. This is of crucial importance as antibiotics resistance of multispecies state to several licensed drugs was proved. Add to this, the increased concentrations of most antimicrobials can lead to undesired side effect. For example the increased concentrations of CHX gluconate, bis- biguanides, quaternary ammonium salts and iodine derivatives are with long-term side effects whose primary is the imbalance of oral ecosystem, also the long term use of 0.2 % w/v CHX can be toxic to gingival fibroblasts, reduce production of collagen and associate with teeth coloration [5, 28, 31]. Several researchers agreed with our study and recorded the superiority of olibanum upon standard antimicrobials. Schillaci and his colleagues (2008) [32] indicated that the extracted active compounds of the essential oil of olibanum showed MBIC values against the 24 hrs. old biofilms of *S. epidermidis* and *S. aureus* at 0.013 and 0.0068 mg/ml respectively below that of the MIC of 0.0226 mg/ml. These data were considered inquisitive since that staphylococcal biofilms are usually resistant to conventional antibiotics at concentrations up to 1000 times the MIC. The publication of Abdoul- latif and his colleagues (2012) [33] recorded that olibanum had antibacterial activity on several G +ve and G -ve isolates stronger than that of some tested antibiotics. Other publication tested the validity of extracted compounds of boswellic acid molecules and recorded MBIC at 16- 32 µg/ml which is in the range of 4X-8X MIC (2- 8 µg/ml) while the effect on mature biofilm was at 32-64 µg/ml (Raja and his colleagues 2011) [18]. In contrast to the current study, the recorded MICs and MBICs in previous ones were lower than the current values; this is because the current study used aqueous crude extract of olibanum while previous studies tested the extracted active compound. The current study proved the validity and determined the effective concentrations of the crude extract of olibanum against the polymicrobial populations of red complex pathogens. These results will be the base for a next complementary experiment utilizes olibanum as a crude extract rather than extracted active compounds as a try to treat polymicrobial periodontal infections.

5. Conclusions

Our results of the effect of antibacterial reagents on different microbial life styles of red complex pathogens can be concluded as follows: The greater effect was on the cells in planktonic growth then on biofilm formation and the lowest effect was on cells in mature preformed biofilm. Each agent well exerted its inhibitory activity on a population of single bacterial type even if existed in a biofilm, however, at higher concentrations. No significant difference of the effect of each reagent between the three types of monomicrobial planktons, but on the biofilms the most significant effect was on *T. denticola*, then *T. forsythia* and later *P. gingivalis*. The effect on

polymicrobial populations was less than that on monomicrobial population; particularly the biofilm involved the two partners, *P. gingivalis* and *T. denticola*. Participation of *P. gingivalis* made the biofilm more resistant. Combination with olibanum encouraged the antibacterial activity of all reagents on all bacterial populations.

6. Limitations of the study

Although the current study proved the *in vitro* benefits of olibanum crude extract to combat the polymicrobial populations of red complex pathogens, but it is best to prove the *in vivo* validity where they are with other synergistic periodontopathogens. However, depending on the current results, it will be possible to conduct a forthcoming study in this regard.

7. Recommendations

The current study recommended for the importance of periodic check for the sensitivity tests of polymicrobial populations of periodontal pathogens to the standard antibiotics to monitor their validity especially against the biofilm. Using olibanum extract with alum in a natural preparation or in combination with CHX or CIP as promising therapy substituting the need for higher concentrations or combination of chemical antibacterials to control periodontal plaque.

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