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Elevation of the Inhibitory Action of Standard Antimicrobials (Ciprofloxacin and Chlorhexidine) by some Natural Materials against Three Periodontal Pathogens

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ABSTRACT

Maintaining the level of periodontal bacteria under control represents the basis for reducing periodontal infections. Therapeutic therapy along with scaling aids to prevent the causative agent from recolonizing the treated surface. As natural substitutes, this study aimed to verify the validity of two natural products, olibanum and alum as inhibitors of periodontal pathogens and also as supporting agents to elevate the anti-action of the pre- validated antimicrobials, ciprofloxacin and chlorhexidine. The study chose three periodontal bacteria, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* as representative taxa because they are considered the more virulent with high proteolytic activity. The antimicrobial activity was studied to find out the minimal inhibitory concentration (MIC) values using resazurin- based microdilution assay. The cooperative interaction between reagents was studied by calculating the fractional inhibitory concentration (FIC) values, analyzing the statistical difference between the single and combinational use and comparing the inhibition zone by agar diffusion. The results proved the inhibitory activity of olibanum and alum against the three pathogens and their high efficacy in improving the inhibitory action of the two standard drugs which was evidenced by the lowered MIC values, calculated FIC values, enlarged inhibition zone and statistical significance of the combinational use. The study concluded the successful use of olibanum and alum in reducing the red complex pathogens either in a single use or in combination as a pure natural preparation and also raising the anti- action of lower concentrations of ciprofloxacin and chlorhexidin.

Keywords: chronic periodontitis, red complex group, natural substances, activity of olibanum, activity of alum.

INTRODUCTION

The control of microbial burden in oral cavity especially the anaerobes periodontopathogens is of crucial role in the course of treating oral infections. Scaling and root planning (SRP) give good results in reducing the burden of etiologic agents for several weeks. The treated site may be recolonized by the pathogen's resident deeper in the pocket or invaded tissues. This emerges the need to use local antiseptics or systematic antibiotics (Tsaousoglou *et al*., 2014). The combinational use of metronidazole with ciprofloxacin or amoxillin and azithromycin has gained popularity as anti- periodontal bacteria (Muhammad[a] and Al-Rawi, 2011; Ananthathavam and Ramamurthy, 2014; Ong *et al*., 2017). The most accepted topical antiseptic with broader activity spectrum than antibiotics to arrest the growth of oral pathogens is Chlorhexidine gluconate (0.12%) (Bogdanovska *et al*., 2012). As safe alternatives, a wide range of different naturals is now under survey as new potential antimicrobials for medical benefits and infections control (Sarker *et al*., 2007). These natural products are environmentally safe, easily available and cheap compared to the toxicity and side effects of allopathic medicines. Therefore, they are promising alternative antimicrobials with therapeutic values for treating human diseases (Vinita *et al*., 2013; Ali, 2018).

One of the interesting natural products, olibanum, is the hardened resins exudate gum from the tree of the genus Boswellia which is also known as frankincense or lubanum and encompasses 20 species. Olibanum consists of essential oils, gum, and terpenoids. It exhibits diverse biological activities. The anti- inflammatory property belongs to the action of boswellic acids in the terpenoid part. The anti-bacterial, anti-fungal and immune-stimulating activity attributes to the essential oil fraction. The resins have been used in the treatment of inflammatory diseases which attributed to its ability to regulate immune cytokines production and leukocyte infiltration (Ismail *et al*., 2014; Sabra and Al-Masoudi, 2014).

The naturally occurring aluminum potassium sulfate "alum" is the crystalized double sulphate aluminum salt with the general formula $KAl(SO_4)_2.12H_2O$. It is odorless, colorless crystalline white solid and soluble in water (Ali, 2018). It has been recommended by the Food and Drug Administration (FDA) as a category for the treatment of burns, ulcers and abscess or as a deodorant and astringent agent. It is a bacteriostatic agent that acts on cell surfaces and interstitial spaces with very low permeability into cells. It has been used for the improvement and preservation of foods, cosmetics; domestic and industrial water treatments (Amadi and Ngerebara, 2017). Alum was also used in water purification as a flocculant and controls bacterial growth by diminishing the *pH* of water. It was considered as harmless material with low toxicity as it isn't absorbed by human body. Nevertheless, high level of alum solution might cause destruction of gum tissues, kidney damage and intestinal bleeding (Bnyan *et al*., 20104; Ali *et al*., 2017). Alum is also used as adjuvant to potentiate the immune response in human (Buonsanti *et al*., 2016)

The scheme of the current study was designed to involve firstly the provability of the inhibitory action of olibanum and alum against periodontal pathogens as a single use and secondly exploring the possible cooperative action of olibanum and alum with the standard antimicrobials used in the course of treating periodontal infections. The tests involved the three correlated pathogens, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, the "red complex group", as they were considered the most predominant etiologic pathogens for chronic periodontitis and more relevant to disease development and severity when their levels exceed a critical threshold leading to destruction of periodontal tissues (Lourenco *et al*., 2014; Torrungruang *et al*., 2015).

MATERIALS AND METHODOLOGY

Bacterial Isolation and Cultivation

Gingival crevicular fluid samples (GCF) were collected from pockets having ≥ 4mm depth and positive bleeding on probe from chronic cases diagnosed in the Teaching Hospital of Dental College at Mosul University. The samples were kept in PBS (*pH* 7.2) till reaching the laboratory where they were cultured into three types of media; Schaedler Anaerobe Agar with blood, haemin,

vitamin K and vancomycin for *P. gingivalis* (Kotsilkov *et al*., 2015), TF medium for *T. forsythia* (Dashper *et al*., 2014), and TYGVS for *T. denticola* (Muhammad[b] and Al-Rawi, 2011). All plates were incubated anaerobically in anaerobic Jar using the microaerophilic atmosphere generation system, CampyGen according to the instructions of the supplier company, Oxiod Ltd, Japan for 5 days at 37°C.

Bacterial Diagnosis

Bacterial isolates were identified morphologically by growth cultural characteristics, microscopically with cell morphology and gram stain as well as by loop mediated isothermal amplification (LAMP) technique.

Natural substance solutions

The natural materials, olibanum and alum were purchased from local perfumer market in Mosul City and tested for their antimicrobial effect on the bacterial isolates. Both were grinded to prepare stock solutions at 0.5 g/ ml for olibanum and 0.6 g/ ml for alum in warmed distilled water (80°C) and dissolved by stirrer. Liqueur extract of olibanum was obtained after overnight soak in warm water.

Standard antimicrobial agents

Stock solutions of two synthetic drugs, 200 mg% CIP (CIPLA LTD, India) and 0.12 % CHX (Scitra Co., UAE, equal to 120 mg% (Wang and Ren, 2017)) were used as a positive control.

Test inhibitory activity of antimicrobial agents as a single therapy

The inhibitory effect was determined in terms of MIC value using resazurin- based microdilution assay (Elshikh *et al*., 2016). Briefly 100 µl of the reagent containing- broth in a double strength was added to column 1 of the 96-well plate. Row A for olibanum, B for alum, C for CHX and D for CIP. From the reagent free- broth medium, 50µl was added to column 2- 11; and 100 µl to column 12 as a control negative to monitor sterility. Reagent containing- broth was two-fold diluted by serial transferring of 50 µl from column 1- 10; the last 50µl from column 10 was discarded. Column 11 served as a positive control for bacterial growth. 50 µl of bacterial suspension was added to each well of column 1-11. The plates were covered and incubated anaerobically in anaerobic Jar using the microaerophilic atmosphere generation system, Campy Gen for 30 hrs. Then, 30 µl of resazurin solution was added to each well from column 1-12 and further incubated for 4 hrs. The result was read as no change of resazurin blue/purple color as indication for inhibition of bacterial growth; or change to colorless- red as indication for bacterial growth. The MIC was the lowest concentration with no change of resazurin color.

Testing inhibitory action of antimicrobial agents in combination use

The interaction between the agents was determined by calculating the fractional inhibitory concentration (FIC) between these gents depending on the procedure of Al-kuraishy and Colleagues (2012) as follows: different concentrations of one antimicrobial agent were combined with different concentrations of the other agents and the FIC was calculated according to the following equation:

(MIC value of drug A in combination / MIC value of drug A alone) + (MIC value of drug B in combination / MIC value of drug B alone)

The FIC indices were interpreted as follows:

< 0.5: synergism, 0.5- 1: additive, 1- 4: indifference, > 4: antagonism.

Agar diffusion method (Muhammad and Al-Rawi, 2011) was also used to compare the inhibition zone of the antimicrobial agent alone and in combination. The absorbance of the growth in microdilution plates was read by a microplate reader at OD_{630nm} and the results were analyzed statistically by one sample *t*- test to find out the significant effect of the single and combinational use within each bacterial type. ANOVA test was also used to find out the significant differences in the single or combinational use between bacterial groups. For each comparison, the significance was considered at p value ≤ 0.05.

RESULTS AND DISCUSSION

The current study tried to explore the interaction manner between the anti- action of two natural products, olibanum and alum, with two standard antimicrobials, CIP and CHX by finding out the FIC values of these reagents. To do so, the MIC of each reagent in a single use was first determined and then the manner of interaction between these reagents was studied. The results of MIC values of these reagents in single use were 1.4- 1.8 mg/ml of olibanum, 1.7- 2.3 mg/ ml of alum, 0.037- .04 mg/ ml CHX and 0.0039 mg/ ml of CHX as are listed in Table (1). CIP and CHX as potent standards purified compounds recorded MIC values lower than that of the aqueous crude of the two natural materials. The statistical analysis of the effect of one reagent alone by one sample *t*- test showed that the reagent has a significant reduction on the cell's growth within each bacterial type. In spite of the differences in MIC values on bacterial types, but according to ANOVA test there were no significant differences in the effect of the reagent between bacterial types. When comparing between all agents by ANOVA test, it was shown that olibanum had the most effect followed by CHX, CIP and alum, also only the effect of olibanum significantly differed from the other reagents, while there was no significant difference between the effects of the other reagents.

Table 1: MIC values of antimicrobial agents against bacterial species in a single use

Bacteria	Agent			
	Olibanum (MIC mg/ml)	**Alum (MIC mg/ml)**	**CHX (MIC mg/ml)**	**CIP (MIC mg/ml)**
P.gingivalis	1.8	2.3	0.04	0.0039
T.denticola	1.4	1.7	0.04	0.0039
T.forsythia	1.4	1.7	0.037	0.0039

In comparison with other studies which tested the extracted active compounds from olibanum (Camarda *et al*., 2007; Al-Saidi *et al*., 2012; Van *et al*., 2010; Mothana *et al*., 2011) they recorded lower MIC values than the present study while in the experiments which also tested olibanum as an aqueous crude, recorded higher MICs value of 8- 25 mg/ml against gram negative (Gr-ve) and positive (Gr+ve) isolates (Al-Kuraishy *et al*., 2012; Ismail *et al*., 2014). On reviewing the previous results, it was obvious that the extracted compound in one study had an anti-effect against a microbial type, but no effect in the others and some extracted molecules displayed less activity against Gr-ve bacteria, but the crude extract of olibanum possesses antimicrobial activity against Gr-ve and Gr+ve aerobes. Studies which tested alum recorded that at 2.5- 15 mg/ml it impeded the growth of several aerobic Gr-ve and Gr+ve bacteria with a significant mean compared with the standard cefotaxime and ofloxacin (Ali *et al*., 2017; Amadi and Ngerebara, 2017). Now, the present study proved the activity of aqueous crude extract of olibanum and alum against the fastidious anaerobic Gr-ve periodontal bacteria, these results will be the basic in the designation of further investigation complementary to the current results which will utilize the aqueous crude extract of olibanum.

In the second part of the study, the MIC value of each reagent in a combinational use with other reagent was determined to calculate the FIC. The results are listed in (Tables 2- 6) which revealed the synergistic inhibition of olibanum with alum, CHX and CIP, also between alum with CHX and CIP as noted by lowering the MIC values in the combinational use in that the FIC values were less than 0.5. By agar diffusion, the inhibition zone of a certain concentration of the reagent was also enlarged in the combinational use. In addition to, the statistical comparison by ANOVA between the single and combinational use on the microbial growth in microdilution plate showed significant superior inhibition of the combinational use and the most significant combination was olibanum with CIP.

Table 2: Combinational interactions between olibanum and alum

Agent / Bacteria	Olibanum (MIC mg/ml)		Alum (MIC mg/ml)		FIC	Interaction
	alone	with alum	Alone	with olibanum		
P.gingivalis	1.8	0.4	2.3	0.3	0.3	Synergisms
T.denticola	1.4	0.3	1.7	0.24	0.3	Synergisms
T.forsythia	1.4	0.3	1.7	0.24	0.3	Synergisms

Table 3: Combinational interactions between olibanum and CHX

Agent / Bacteria	Olibanum (MIC mg/ml)		CHX (MIC mg/ml)		FIC	Interaction
	Alone	with CHX	Alone	with olibanum		
P.gingivalis	1.8	0.3	0.04	0.006	0.3	Synergism
T.denticola	1.4	0.22	0.04	0.006	0.3	Synergism
T.forsythia	1.4	0.22	0.037	0.006	0.3	Synergism

Table 4: Combinational interactions between olibanum and CIP

Agent / Bacteria	Olibanum (MIC mg/ml)		CIP (MIC mg/ml)		FIC	Interaction
	Alone	with CIP	alone	with olibanum		
P.gingivalis	1.8	0.22	0.0039	0.0004	0.2	Synergism
T.denticola	1.4	0.22	0.0039	0.0003	0.2	Synergism
T.forsythia	1.4	0.17	0.0039	0.0003	0.2	Synergism

Table 5: Combinational interactions between alum and CHX

Agent / Bacteria	Alum (MIC mg/ml)		CHX (MIC mg/ml)		FIC	Interaction
	Alone	with CHX	Alone	with alum		
P.gingivalis	2.3	0.3	0.04	0.007	0.3	Synergism
T.denticola	1.7	0.25	0.04	0.009	0.36	Synergism
T.forsythia	1.7	0.25	0.037	0.007	0.32	Synergism

Table 6: Combinational interactions between alum and CIP

Agent / Bacteria	Alum (MIC mg/ml)		CIP (MIC mg/ml)		FIC	Interaction
	Alone	with CIP	Alone	with alum		
P.gingivalis	2.3	0.37	0.0039	0.0006	0.3	Synergism
T.denticola	1.7	0.37	0.0039	0.0006	0.35	Synergism
T.forsythia	1.7	0.37	0.0039	0.0006	0.35	Synergism

In the course of periodontitis treatment, CHX is recommended as a local antiseptic at a concentration of 120 or 200 mg% according to the severity of the infection. The concentration will be higher at time of application, but the level will be diminished after fewer later. The prolong use of CHX has also a toxic effect on gingival fibroblast (Lakshmi *et al*., 2014). As a systemic administration, the oral dose of 500 mg of CIP is prescribed twice daily which reaches human GCF in a mean level of 0.00338 mg/ml after 2hr. and 0.00124 mg/ml after 7 hr. (Dincel *et al*., 2005) which are lower than the MIC value recorded in the current study, 0.0039 mg/ml. To show

therapeutic benefits on anaerobic bacteria, systematic antibiotics are prescribed in higher dose along with metronidazole which can cause gastric upset. The current study shows the reproducibility use of the two natural materials in combination of each other or with the standard antimicrobials to exert the demand benefit. This was proved by the lowered MICs in the presence of natural products; and also, the enlarged diameter of inhibition zones. This terminates the need of increasing the concentrations of standard antimicrobials or use two types of antibiotics and overcomes the side effects of prolong exposure of these antimicrobials. The current work achieved its goals in proving the effectiveness of the crude extract of the two natural agents against the red complex pathogens and the synergistic inhibition with the standard drugs. These results will be starting point for the next complementary experiments depend on the crude extract to compact the mixed bacterial infection of periodontitis.

The multifunctional constituents of the crude extract of olibanum correlated with its activity. Boswellic acids in the terpenoid fraction exert anti- inflammatory effect. Resin fraction was described to destroy microbial cell wall and stop protein synthesis. Sticky nature of water- soluble gum stops the reactions between substances (Sabra and Al-Masoudi, 2014). Keto-ß-boswellic acid was reported to distort the cell membrane structure, and disrupt the permeability barrier of microbial membrane structures. Olibanum was also reported to be a DNA intercalator and inhibits bacterial DNA synthesis through topoisomerase inhibition (Al-kuraishy *et al*., 2012). Alum acts by reducing the acidity leading to unfavorable environment for bacterial growth and causing precipitation of proteins or deleterious effects on bacterial cell wall (Amadi and Ngerebara, 2017; Ali, 2018). By these mechanisms of activity these naturals had enhanced the action of CIP in ceasing cell replication, and CHX in disrupting cell membrane.

The findings of several previous researches support the concepts of present study. Horiuchi and his team (2007) recorded that olibanum synergistically improves the action of aminoglycosides against vancomycin resistant enterococci; and the synergism between olibanum extract and tetracycline, chloramphenicol and ciprofloxacin were observed by the team of Aqil (2007). Also, the study of Al-Kuriashy and his colleagues (2012) found by using FIC method that olibanum boosted the anti-action of clarithromycin on five Gr-ve and three Gr+ve isolates by lowering the MIC value with a significant difference than if used alone. These studies acclaimed the synergistic capacity of plant extracts to improve the antibiotics activity and considered olibanum as a modifying agent by lowering the MIC values of the antibiotics or modifying membrane fluidity and increased permeability of antibiotics even in resistant bacteria. Bnyan *et al*., (2014) found that 20% (w/v) alum had significantly increased the anti- action of CHX and erythromycin rather than a single use against selected aerobic pathogenic bacteria. Also, the researcher Ali (2018) published that 10% alum had strong inhibition against *S. aureus*, *E. coli* and *K. pneumonia* by increasing the efficacy of tetracycline and cefotaxime as indicated by increased diameter of inhibition zone better than when the tow antibiotics were combined.

CONCLUSION

The current study indicates the effectiveness of olibanum crude extract and alum to control the infections of periodontal bacteria as a single use or in combination as a pure natural agent as they significantly inhibited the bacterial growth. It is also of significant value using these natural agents as local antibacterial in the formula of oral dentifrices and CHX mouth rinses or adjustment to systemic antibiotics in the course of treatment to obtain superior results with no need to increase the concentrations or combining the standard drugs.

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رفع الفعل التثبيطي للمضادات القياسية (Ciprofloxacin وChlorhexidine) بواسطة بعض المواد الطبيعية ضد ثلاث ممرضات انسجة ما حول الاسنان

الملخص

ابقاء اعداد الجراثيم في انسجة ما حول الاسنان تحت السيطرة يمثل اساس تقليل اصابات انسجة ما حول الاسنان. استخدام الجرع العلاجية بعد التنظيف الميكانيكي للترسبات تحت اللثة يساعد في منع اعادة استعمار الجراثيم المسببة. تهدف الدراسة الحالية الى اثبات تاثير نوعين من المواد اللبان والشب كبدائل طبيعية لتثبيط ممرضات ماحول الاسنان وكعوامل داعمة لفعل دوائين قياسيين Ciprofloxacin وChlorhexidine. تم اختيار ثلاث انواع من الجراثيم هي *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* والتي تعتبر من أكثر ممرضات ما حول الاسنان ضراوة. حددت الفعالية ضد المايكروبية بايجاد قيمة التركيز المثبط الادنى باستخدام طريقة Resazurin- based microdilution assay. التعاون التازري لفعل المواد المضادة درس عن طريق حساب قيمة Fractional inhibitory

concentration (FIC) وبتحليل الاختلاف الاحصائي بين الاستخدام المفرد والمتزامن للمواد وكذلك بمقارنة قطر منطقة تثبيط النمو بطريقة الانتشار بالاكار. اثبتت النتائج الفعالية التثبيطية للبان والشب ضد الممرضات الثلاث وكذلك كفاءة هذه المواد الطبيعية لرفع فعالية الادوية القياسية والذي استدل عليه بتقليل التركيز المثبط الادنى وقيمة FIC المحسوبة وكذلك اتساع منطقة تثبيط النمو والمعنوية الاحصائية عند الاستخدام المتزامن للمواد المضادة. استنتجت الدراسة الحالية كفاءة استخدام اللبان والشب في اختزال ممرضات "red complex"سواء عند استخدامها بشكل مفرد او سوية كمستحضر مواد طبيعية او استخدامها مع Ciprofloxacin و Chlorhexidine لرفع الفعالية المضادة للتراكيز الاقل من هذه الادوية.

الكلمات الدالة: التهاب انسجة ما حول الاسنان المزمن، مجموعة red complex، المواد الطبيعية، فعالية اللبان، فعالية الشب.