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Effect of Lubanum and Potash Alum on Co-aggregation and Biofilm Formation of *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*

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ABSTRACT

Porphyromonas gingivalis, *Treponema denticola*, and *Tannerella forsythia* can co- aggregate in a more stable and resistant multispecies biofilm. Therefore, the current study aimed to test the effectiveness of lubanum and potash alum solutions, as a natural rinse, to affect the attachment between the cells and the multispecies biofilm of these periodontitis pathogens, and also to enhance the action of the chemical rinse, BioFresh K (chlorhexidine (CHX)). The results revealed that the solutions of 12 mg/ ml of both natural substances can prevent bacterial co-aggregation, and the solutions of 40 mg/ ml of natural substances and 1.2 mg/ml of CHX can affect the cells in their polymicrobial population after 1hr exposure. The results also revealed that increasing the concentration of lubanum made it possible to affect the resistant preformed multispecies biofilm and the effect became more maxima when it was used in combination with other agents. This combination is benefit in controlling the chronic periodontitis caused by these pathogens as it can reinforce the effect of antibacterial agents on multispecies biofilm and prevent the attachment of new cells without the need of increasing the concentration or combination between chemical therapies.

Keywords: Co- aggregation, multispecies biofilm, periodontal pathogens.

INTRODUCTION

The pathology of periodontitis is characterized by co-adherence of different anaerobic types to promote the formation of a multispecies subgingival biofilm (Chaudhary *et al*., 2020). Presence of these pathogens within a multispecies biofilm endows them more stabilization and resistance to washing and antimicrobial agents because more extracellular matrix is produced thus become more cohesive with each other and fixed to the surface; this matrix also provides physical barrier which impedes the diffusion of antimicrobial factors (Raja[a] *et al*., 2011; Wang and Ren, 2017). Therefore, chemical antimicrobials are less effective against biofilm which demands the use of higher concentration or combination of antimicrobials in the treatment (Bogdanovska *et al*., 2012; Belibasakis and Thurnheer, 2014; Ong *et al*., 2017). These resolutions will seriously increase the occurrence of side effects to human and the emergence of new resistance because cells within microbial biofilm are with high frequency of exchange of resistance genes and mutations (Raja[b] *et al*., 2011; Wang and Ren, 2017; Tsaousoglou, 2014). The apparent example is the biofilm of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*; the most putative causatives of chronic periodontitis, which are also known to be the most cooperative pathogens that coaggregate into a subgingival multispecies biofilm resistant to chemotherapy. (Kotsilkov *et al*., 2015). The inefficiency of chemical therapeutics leads the scientists to search for new antimicrobials that are more effective against microbial biofilms and safe at high concentrations to avoid side effects on human. Therefore, the aim of our study was to search the efficiency of two natural substances, lubanum and potash alum, as substitutes or supporter to the chemotherapy in affecting co- aggregation and multispecies biofilm of these pathogens.

MATERIAL AND METHODS

Preparation of aqueous extracts and solutions

The powder of lubanum (*Boswellia* sp.) and potash alum (potassium potash alum) was dissolved in warmed water at 80°C to prepare solutions at a concentration indicated in each experiment. Primary solution of the mouth rinse Biofresh K (Scitra Co., UAE) was used at 0.12% w/v of chlorhexidine digluconate (1.2 mg/ ml CHX). Primary solution of Ciprofloxacin (CIPLA LTD, India) used was 2 mg/ ml. Co-aggregation buffer (CAB) was prepared as stated by Sato and Nakazawa (2014) to contain 1 mM Tris- HCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 mM NaCl, and 0.02%NaN_3 with pH = 7.8. Solutions of the two dyes of the LIVE/DEAD® BacLight Bacterial Viability (cat. L13152) were prepared following the company commands (Molecular Probe Inc., Invitrogen, USA).

Bacterial isolates

Gingival fluid of chronic periodontitis was sampled as a source of the three types of bacteria. Collection, transportation and culturing of samples on the selective medium (Schaedler Anaerobe blood Agar for *P.gingivalis*, TYGCS for *T. denticola* , and TF agar for *T. forsythia*) were conducted according to Al- Hamdoni and Al- Rawi (2020)[a]. Bacterial types were also purified and confirmed following the authors' publication.

Effect of natural substances on the coaggregation among bacteria

This experiment was designed as mentioned elsewhere (Sato and Nakazawa, 2014) to study the effect of several concentrations (2-12 mg/ml) of lubanum and potash alum on the coaggregation among bacteria. Bacterial cells were cultivated for 3-days then washed and suspended in a CAB (equal to McFarland tube 5). Three sets of tubes were prepared: tubes of a positive control set contained 1 ml from a suspension of one bacterial + 1ml of CAB + 1ml from a suspension of other bacterial type; tubes of a test set contained 1 ml from a suspension of one bacterial +1ml of lubanum or potash alum + 1ml from a suspension of other bacterial type; tubes of a negative control set contained 1ml of lubanum or potash alum + 2 ml of CAB. All tubes were kept standing with shaking at ambient temperature. Coaggregation between cells was scored as 0 for no evident

aggregates and unaltered turbidity; 1 for the fine aggregates diffused in a turbid solution; 2 for the evident aggregates that did not precipitate immediately; 3 for the settled aggregates with a somewhat turbid supernatant; and 4 for the settled aggregates in a clear solution.

Effect of the antibacterial agents on the multispecies cell population and biofilm of three types of bacteria

The effect of a single or combinational use of 40 mg/ml lubanum and potash alum, 1.2 mg/ml CHX and 2mg/ ml CIP on the multispecies cultures was evaluated after one hour of exposure. Cells were stained by the fluorescent dye of the LIVE/DEAD® BacLight Bacterial Viability to estimate the effect of this treatment.

1. The multispecies cell population and biofilm cultures of the three bacteria

Multispecies cell and biofilm population was prepared following Yamada *et al*., (2005) and Park *et al*., (2014). Briefly, a slide cover was placed in a small Petri dish to which a total of 6ml of a mixture of an antibiotic- free media was poured (2 ml from an uncultured medium for each type of bacteria). Then, 0.1 ml from a suspension of each type of bacteria (compared with McFarland 0.5) was added to this mixture of media. After 4 days of anaerobic incubation, the liquid growth was the multispecies cell population and the growth on the slide cover was the multispecies biofilm.

2. The effect on the multispecies cell's population

Following the company instruction, multispecies cell population was precipitated from the liquid growth by a centrifuge (3000 rpm for 10 min), washed, re-suspended in 1 ml of normal saline, mixed with 3 ml an antibacterial agent and incubated with shaking at ambient temperature for 1 hr. To the control sample, normal saline was added. Then, all samples were centrifuged (3000 rpm for 10min) and the cells were washed, re-suspended in 1 ml of normal saline, mixed with 1 ml of the 2X mixture of the fluorescent dyes and incubated for 15 min in dark at room temperature. Then, 5µl of the stained bacteria was placed on a slide and covered with coverslip and examined by a fluorescence microscope.

3. The effect on the multispecies biofilm

Following the team of Park (2014), the multispecies biofilm on the slide cover was washed with normal saline, 3 ml of the antibacterial agent (alone or in combination with lubanum) was added and incubated with shaking at ambient temperature for 1 hr. To the control sample normal saline was added. Then, all samples were washed with normal saline, stained with 0.1 ml of 1X mixture of the fluorescent dyes for 15 min. in dark at room temperature, washed and examined by a fluorescent microscope.

RESULTS AND DISCUSSION

Effect of natural substances on the coaggregation between bacteria

Coaggregation of *P. gingivalis* with *T. denticola* or *T. forsythia,* and *T. denticola* with *T.forsythia* was noticed within short time of mixing with each other and it was so evidently clear between *T. denticola* and *P. gingivalis*. These bacteria adhered quickly to each other forming sediment of clumps in a clear solution as it is obvious in the positive control (tube A) in Fig. (1) without lubanum or potash alum. Stained smear from this tube in Fig. (2) shows cells of *P. gingivalis* attached to *T. denticola*. Gradually increased concentrations (2 to12 mg/ ml) of lubanum or potash alum lowered the score of co-aggregation gradually to 2 (tube D) and to 1 (tube C) and finally to 0 (tube B with the highest concentration). The score was assigned after comparing with the positive and negative controls. These results were observed with the three types of pathogens in the presence of lubanum or potash alum.

Fig. 1: Coaggregation between *P.gingivalis* and *T.denticola.* (A): positive control for coaggregation. In the presence of lubanum the score was: 0 for no aggregation and unaltered turbidity (B), 1 for the fine aggregates diffused in a turbid solution (C) and 2 for the evident aggregates that did not precipitate immediately (D).

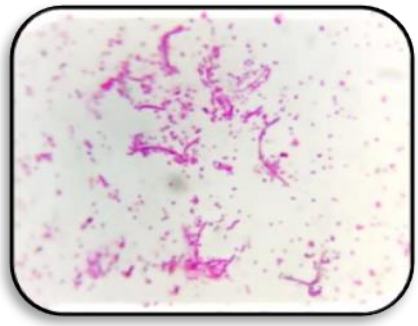

Fig. 2: *T.denticola* cells co-aggregated to *P.gingivalis*. Cells were stained with carbolfuchsin and examined by light microscope

Co-aggregation between periodontal pathogens is one of the pathogenicity phenomena by which the weak colonizer attaches to the former one, this interconnection is a fundamental step for the establishment of the multispecies biofilm on tooth surface or subgingival tissues (Saito *et al*., 2008). Therefore, the study was interested in this interaction and confirms the practical benefits of lubanum and potash alum to block this phenomenon. The study suggests that the two natural substances may distort bacterial cell structures that promote the co- adherence among periodontal bacteria. Co-aggregation was recorded to occur among different species of oral bacteria involving *Actinomyces naeslundii, A. viscosus, Streptococcus gordonii, Str. mutans, Str. oralis, P. gingivalis, Prevotella intermedia, P. oris, Fusobacterium nucleatum, T. denticola, T. medium.* Self-aggregations do not occur within type except for *F. nucleatum* and *A. viscosus* (Yamada *et al*., 2005; Tan *et al*., 2014). The gingipaine adhesin, proteineous structures on the surface of *P.gingivalis* mediate co-aggregation to dentillisin on the surface of *T. denticola* within 15-40 min and to the glycosylated proteins of the S- layer of *T forsythia* (Ito *et al*., 2010; Sharma, 2010; Zhu *et al*., 2013; Sato and Nakazawa, 2014).

Effect of the antibacterial agents on multispecies cells population and biofilm of the three pathogens

The results of these experiments revealed that one hour exposure to 1.2 mg/ ml of CHX and 40 mg/ ml of lubanum and potash alum was effective against the multispecies cells population, while 2mg/ ml of CIP was ineffective Fig. (3). On the other hand, such treatments were ineffective against

the pre- established multispecies biofilm; however, the effect started upon increasing the concentration of lubanum with time of exposure or if 200mg/ ml of lubanum was used together with potash alum or CHX Fig. (4). The outcomes of these treatments were displayed using the mixture of the nucleic acid fluorescent dyes before and after exposure. These dyes are useful for the quick evaluation of the effect because they rapidly define the viability of bacteria. The green fluorescent dyes, SYTO® 9 green commonly labels all cells in a population- those with integral or damaged membranes while the red-fluorescent dye, propidium iodide can only penetrate cells with damaged membranes, causing a reduction in the fluorescence of SYTO® 9 green dye.

The untreated (positive controls) cells and biofilm in a multispecies existence shined with green fluorescent under the fluorescence microscope as in Fig. (3 A and 4 A) respectively. Treatment with the above specified concentrations of lubanum, potash alum and CHX for one hour was effective against the cells mixture which was apparent by increasing the red fluorescence of propidium iodide and reduce in the green fluorescence of SYTO® 9 green Fig. (3 B, C and D). However, treatment with CIP was not effective as cells remained intact with green fluorescence Fig. (3 E). Treatment with each agent alone was also not so effective against pre- established multispecies biofilm. Therefore, we tested the advantage of increasing the concentrations of lubanum till 200 mg/ ml and the time of exposure; in addition to the combination with CHX and potash alum which showed an inhibitory effect against biofilm as the green fluorescein reduced and the red fluorescence increased Fig. (4 B-D). CHX was not used higher than 0.12% because the principal purpose of the study was to find natural alternatives to the increased concentrations of chemical antimicrobials to avert side effects. Also, potash alum was not planned for use at higher concentrations as earlier references cited that higher concentrations may unfavorably affect tissues of gum, kidney and intestine (Al- Talib *et al*., 2016; Ali, 2018). CIP was not involved in testing the combination with lubanum because even its high concentration was ineffective against free cells during 1hr and sensibly it will be ineffective against cells embedded in the biofilm; and upon combination the action will be only related to lubanum.

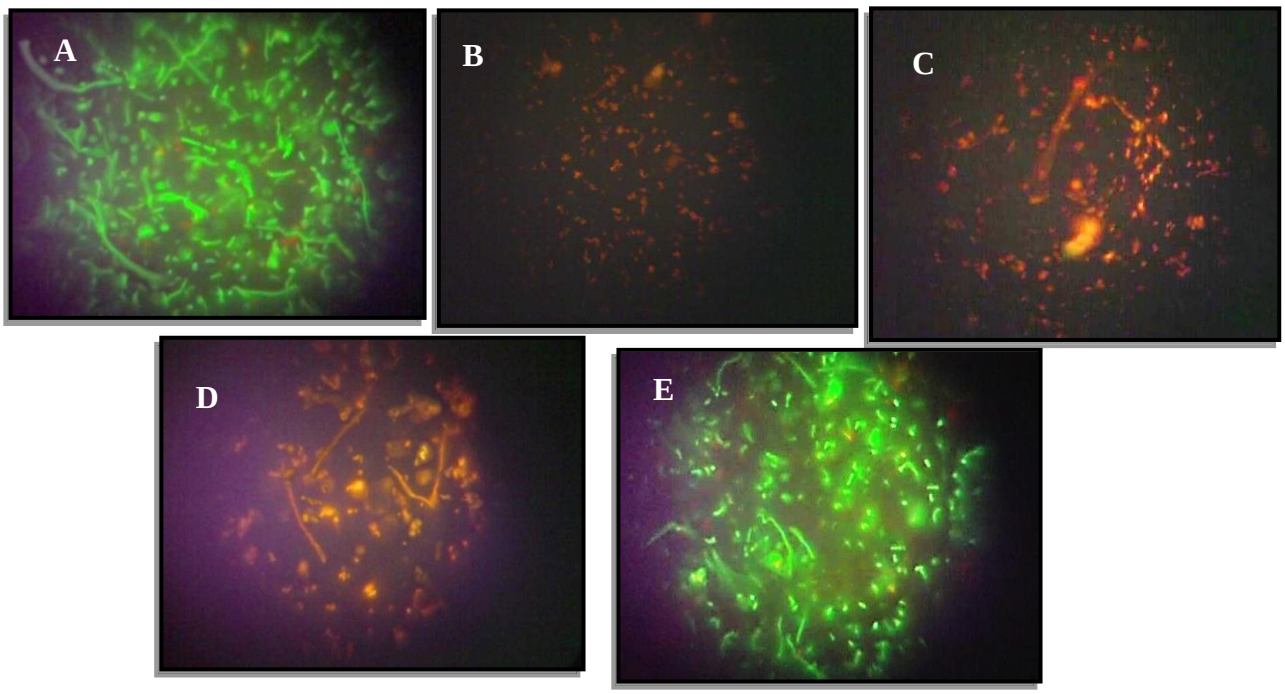

Fig. 3: Fluorescein microscope images (1000 X) of the multispecies cell's population of *T. denticola, P. gingivalis* and *T. forsythia*. (A): lived green fluorescent cells. Dead red cells exposed to: (B) lubanum, (C) potash alum, (D) CHX. (E): lived green fluorescent cells exposed to CIP

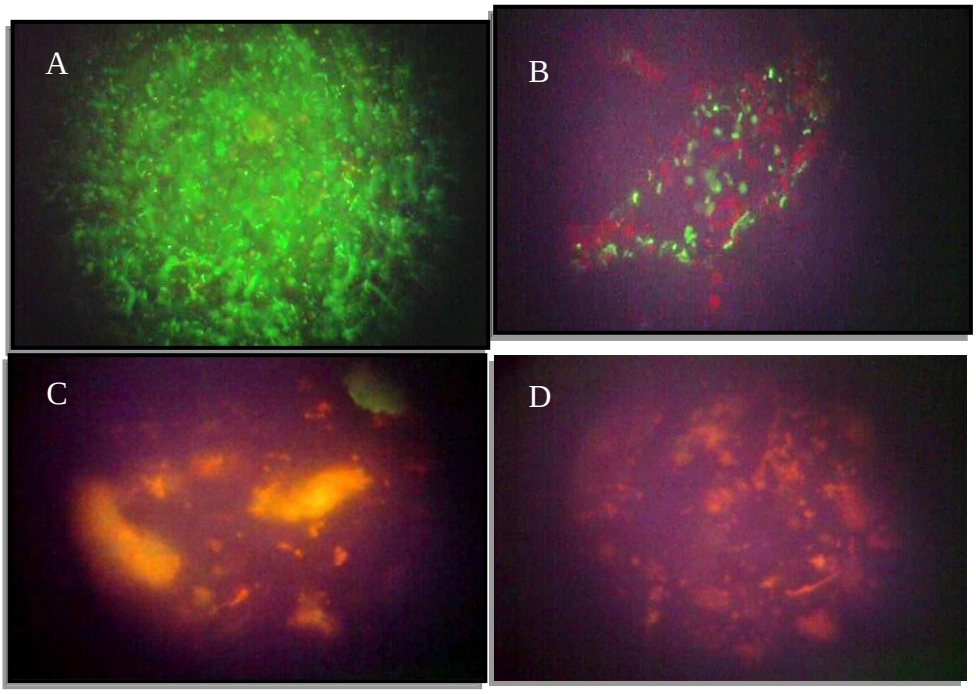

Fig. 4: Fluorescein microscope images (1000 X) of the multispecies biofilm of *T. denticola, P. gingivalis and T. forsythia*. (A): untreated with green fluorescent, (B): dead red and lived green treated with lubanum. Dead red treated with (C) lubanum and CHX, (D) lubanum and potash alum

In our earlier experiment Al-Hamdoni and Al-Rawi, 2020[b], we have assessed the effect of antimicrobial agents on the growth of multispecies cells and biofilm after 24- 72 hrs. exposure. We have recorded that the growth of multispecies cells population was inhibited by lubanum, potash alum, CHX and CIP, the formation of multispecies biofilm was inhibited by only lubanum and CHX, and the pre- established multispecies biofilm was inhibited by only 30mg/ ml of lubanum while 37.5 mg/ml of potash alum, 1.2 mg/ml of CHX and 2 mg/ml of CIP were ineffective. In addition, the use of lubanum with other agents strengthened the inhibitory effect of all agents. The current experiment was planned to expose the multispecies population of cells and pre- established biofilm of the three periodontitis bacteria to antibacterial agents for one hour as similar as the use of local antiseptics, therefore, the agents were used at concentrations higher than the MIC and MBIC documented in the earlier experiment. Therefore, CHX was used at 1.2 mg/ ml (0.12% w/v, the concentration of a commercially available); potash alum and lubanum were used at 40 mg/ ml.

CONCLUSIONS

The current study can document the following inclusions: (1) Lubanum and potash alum can affect the co-aggregation among *T. denticola, P. gingivalis and T. forsythia.* The use of both naturals in a mouth rinse can help to prevent bacterial accumulation in the biofilm. (2) The effect of antibacterial agents within one hour can be evaluated using fluorescent dyes, which can give reliable indication to assess the spectrum of the effect of the topical rinses. (3) Antibacterial agents showed the effect during such treatment were those affect the integrity of cell envelop, CHX, lubanum and potash alum; while CIP which effects multiplying cells showed no effect during this period. (4) Increased concentrations of lubanum showed activity against cells in the biofilm within 1 hr. which along with the safety use for human consumption open the road to design a next experiment for using lubanum as a medicinal product for the treatment of oral infections. (5) Combination reduces time required to display the demand effect.

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تأثير اللبان وشب البوتاس على التجمع وتكوين الغشاء الحيوي للأنواع *Porphyromonas gingivalis* و *Tannerella forsythia* و *Treponema denticola*

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الملخص

تتجمع خلايا الانواع *Porphyromonas gingivalis*، *Treponema denticola* ، و *Tannerella forsythia* مع بعضها ضمن غشاء حيوي متعدد المايكروبات اكثر ثباتاً ومقاومة. لذلك تهدف الدراسة الحالية الى اختبار كفاءة محاليل اللبان وشب البوتاس، كغسول فم من مواد طبيعية، في التأثير على الالتصاق بين الخلايا وعلى الغشاء الحيوي متعدد المايكروبات لممرضات انسجة اللثة الثلاثة، ايضا في تعزيز فعل الغسول الكيميائية (BioFresk K (Chlorhexidine (CHX)). أظهرت النتائج ان محاليل 12 ملغم/ مل من المادتين الطبيعيتين ممكن ان تمنع حدوث التجمع البكتيري وان محاليل بتركيز 40 ملغم/ مل من المواد الطبيعية و 1.2 ملغم/ مل من غسول CHX ممكن ان تؤثر على خلايا الممرضات في مجتمعها المتعدد المايكروبات بعد التعرض لمدة ساعة. اظهرت النتائج ايضا ان زيادة تركيز اللبان جعل من الممكن التأثير على الغشاء الحيوي متعدد المايكروبات الناضج وأصبح التأثير أكبر عند استخدام مزيج اللبان مع العوامل المضادة الاخرى. استخدام هذا المزيج كغسول فم يكون ذو فائدة في السيطرة على اصابات انسجة حول الاسنان المزمنة الناجمة عن هذه الممرضات اذ انه يعزز تأثير العوامل المضادة للبكتريا على الغشاء الحيوي المتعدد المايكروبات ويمنع التصاق خلايا جديدة مع الغشاء الحيوي وبدون الحاجة الى استخدام تركيز اعلى او مزيج من العوامل الكيميائية الضد مايكروبية.

الكلمات الدالة: التجمع، الغشاء الحيوي متعدد المايكروبات، ممرضات انسجة حول الاسنان.