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OPEN PEER REVIEW

In vitro Evaluation of Synergistic Inhibitory Effect of Propolis and Chlorhexidine on Biofilm Cells of Oral Pathogens

Hadis Tavafi

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1 Original submission

2 comments · 1 Jan 2022 – 12 Jan 2022

Morteza Saki Reviewer

MAJOR REVISION 1 Jan 2022

The current study aimed to evaluate the synergistic inhibitory effect of propolis and chlorhexidine on biofilms of some oral bacteria. Although the work is interesting, there are several issues that need to be revised, clarified, or corrected to improve the manuscript, as follows:
Important note: First and foremost, this article needs general grammatical correction, especially in the methods section. I marked some of the errors in red in the attached file.
Title
- Change the title of the manuscript to: In vitro evaluation of the synergistic inhibitory effect of propolis and chlorhexidine on biofilm cells of oral pathogens
Abstract
- Write the date when the experiment was performed in the methods section.
- Write the collection source or origin of the studied bacteria (standard or clinical) in the methods section. Also, add the percentage for CHX.
- Write the results of MBIC and MBEC for propolis, chlorhexidine, and their combination in the results section.
- Delete antifouling from the conclusion.
Background
- Line 3, page 1, complete the sentences related to: “by various factors such as environmental factors, the use of antibiotics, and...”
- Use relevant references for the first sentences of the first paragraph. There are several sentences without references.
- Divide the first paragraph into smaller parts.
- The authors claimed that Staphylococcus aureus is among the most common oral biofilm-producing pathogens. Clarify this with an appropriate reference.
- Add the Objectives subheading after the background.

Materials and methods
- Change Materials and methods to Methods.

- Add the reference numbers for CLSI 2019 and Tavafi et al., 2018.
In CLSI 2019, I can’t see the relevant inhibition zone for metronidazole for the studied bacteria. Also, amoxicillin was not in the CLSI list for Staphylococcus. I recommend deleting this part, “In order to evaluate the susceptibility of selected standard strains to common antibiotics (amoxicillin, penicillin, metronidazole) in the treatment of oral infections, a disk diffusion test was performed and the sensitivity and resistance to antibiotics according to the CLSI M100-standard method. S29 was determined (CLSI, 2019)”, from the manuscript, as it does not add anything to the content of the manuscript.
- Add the date of the study in the methods section.
- Add the source (manufacturer name, country) of propolis and chlorhexidine. Also, add the manufacturer name and country for all materials and devices used in the study, such as TSB, TSA, TTC, and ELISA Plate Reader.
- The diluent for propolis is not mentioned. Also, the authors did not clarify whether propolis was used directly or its extract was examined.
- The materials and methods section lacks fluency in its presentation of the content, which makes it difficult to understand. Also, there are very obvious writing and grammatical errors. Rewrite the full section.

Results
- As noted in the previous comment, I recommend deleting the first paragraph related to antibiotic disk diffusion.
- Move “the percentage of inhibition of biofilm formation in each case was calculated using the following formulas” and the related formula to materials and methods.
- Write the main results, such as the MBIC values for propolis and CHX 0.2%, the MBEC results for propolis and CHX 0.2%, the results of the synergistic effect of propolis and CHX 0.2% on MBIC, and the results of the synergistic effect of propolis and CHX 0.2% on MBEC, in the appropriate sections.
- The authors claimed that they used a P value of <0.05 to indicate significant results, but these analyses were not reflected in any part of the results.
Discussion
- The first 4 paragraphs are not necessary and can be written as one paragraph.

- Write about the limitation of the current study, such as the lack of a method for the chemical analysis of the compounds in propolis, such as gas chromatography–mass spectrometry (GC-MS).

References
- Check all references to ensure that all of them follow the journal style. Some references seem to be incorrect. Also, in the main text, the references do not follow the journal style, and the authors used separate parentheses for each reference, such as (18)(19). Instead, they need to be written as follows: (18,19). Moreover, in some parts, the citations are not in the appropriate order, such as (13)(11) and (21)(2). Check and correct all text accordingly.

Tables
All tables can be designed as one table. I recommend writing the results in the text and making the tables more concise.

Editor-in-Chief MAJOR REVISION 12 Jan 2022

Dear Dr. Hadis Tavafi
Thank you for considering JJCMB for publication of your manuscript entitled "Evaluation of the inhibitory synergistic effect of propolis and chlorhexidine on biofilm cells of oral pathogens in vitro". Your manuscript needs a major revision. To avoid an extra revision, follow the forwarded comments and revise your manuscript meticulously and point by point.
Best Regards

EIC, Dr. Mard

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Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Reviewers' Comments:

All comments were applied to the original text and highlighted.

Reviewer 1:
The current study aimed to evaluation the synergistic inhibitory effect of propolis and chlorhexidine on biofilm of some oral bacteria. Although the work is interesting, there are several issues that need to be revised, clarified, or corrected to improve the manuscript as follows:
Q1: Important note: First and foremost, this article needs a general grammatical correction, especially in the method section. I mentioned some of the errors in red color in attached file.

R1: The whole text was grammatically re-examined and the deficiencies were eliminated.

Title
Q2: Change the title of the manuscript to: In vitro evaluation of the synergistic inhibitory effect of propolis and chlorhexidine on biofilm cells of oral pathogens

R2: Lines 1&2: Title changed to: In vitro evaluation of the synergistic inhibitory effect of propolis and chlorhexidine on biofilm cells of oral pathogens

Abstract
Q3: Write the date of experiment performing in the method.

R3: line 10: was written the date of experiment performing in the method. (Performed Oct. 2021)
Q4: Write the collection source or origin of studied bacteria (standard or clinical) in the method section. Also, add the percent for CHX.

R4: Lines 12 &13: In this study (performed Oct. 2021), the minimum biofilm inhibitor concentrations (MBIC) and the minimum biofilm eradication concentrations (MBEC) of CHX 0.2% and propolis aqueous extract were determined against *S. mutans* and *S. aureus* (were prepared from the cell bank of Pasteur Institute of Iran).

Q5: Write the results of MBIC and MBEC for propolis, chlorhexidine, and their combination in the result section.

R5: Lines 16-21: The results showed that the MBIC of chlorhexidine for both *S. aureus* and *S. mutans* was 125 µg/ml and the MBIC of propolis aqueous extract was 937.5 and 3750 µg/ml, respectively. Also, MBEC chlorhexidine for *S. aureus* and *S. mutans* was 250 and ≥500 µg/ml and MBEC propolis 1875 and ≥7500 µg/ml, respectively. The results showed that combining the two substances showed a

synergistic effect in inhibiting biofilm formation ($p \leq 0.05$) while they had an indifferent effect on biofilm eradication.

Q6: Delete the antifouling from conclusion.

R6: Line 24: Deleted the antifouling from conclusion.

Background
Q7: Line 3, page 1, complete the sentences related to: “by various factors such as environmental factors, the use of antibiotics, and...”

R7: lines 31 &32: In this type of disease, the microbial flora of the mouth is altered by various factors such as environmental factors and the use of antibiotics.

Q8: Use the relevant references for the first sentences of first paragraph. There are several sentences without references.

R8: lines 30- 35: Used the relevant references for the first sentences of first paragraph. (1).

Q9: Divide the paraghraph one to smaller parts.

R9: lines: 30- 51. Divided the paragraph one to smaller parts.

Microorganisms are the most important causative agents of common oral diseases such as caries and periodontal diseases. In this type of disease, the microbial flora of the mouth is altered by various factors such as environmental factors and the use of antibiotics. Microorganisms in the oral cavity can communicate by producing their metabolic products or exchanging molecular signals. Due to these intercellular connections of microorganisms, the possibility of microbial biofilm formation also increases (1).

Biofilm formation is one of the most important factors in tooth decay. A microbial biofilm is a community of microbes stacked together in an extracellular matrix of polysaccharides. Microbial biofilm is one of the most important causative agents for many oral infectious diseases, including tooth decay, gingivitis, periodontitis, periapical periodontitis, and peri-implantitis (1, 2). Controlling the formation and inhibition of oral biofilm causes high costs for patients (3). The biofilm formation cycle includes bacterial binding, biofilm growth/maturation, and biofilm dispersion. Each of the biofilm formation stages can be inhibited, and solutions that can disrupt each step of the biofilm cycle are a possible way to control the biofilm (4).

It is always easier to deal with pathogens in the form of planktonic cells compared to pathogens in adult biofilm, and higher concentrations of antimicrobial agents are not required to eradicate these agents (5, 2), But the matrix in the biofilm reduces the availability of the drug and prevents the drugs from penetrating its deep layer (6, 7). This feature of biofilm has caused even the most effective methods of controlling oral diseases to fail due to the increased resistance of biofilms to antibiotics and other antimicrobial agents (8, 9).

Q10: The authors claimed that Staphylococcus aureus is among the commonest oral biofilm-producing pathogens. Clarify this with an appropriate reference.

R10: Line 55: ref (10).

Q11: Add the Objectives subheading after background.

R11: line 29: Background & *Objectives* and lines99-105: This study aimed to investigate the synergistic effect of the inhibitory anti-biofilm activity of CHX and propolis aqueous extract on common oral biofilm-producing pathogens, *Streptococcus mutans*, and *Staphylococcus aureus*. Minimum biofilm inhibitory concentrations (MBIC) and minimum biofilm eradication concentrations (MBEC) of CHX and propolis against these microorganisms were determined, and then, the synergistic effect of these two compounds on inhibiting the formation and biofilm of the studied bacteria in vitro were evaluated using the checkerboard method.

Materials and methods
Q12: Change the materials and methods to Methods

R12: line 106: Changed the materials and methods to Methods

Q13: Add the reference number for CLSI 2019 and Tavafi et al., 2018.
In the CLSI 2019, I can't see the relevant inhibition zone of metronidazole for studied bacteria. Also, the amoxicillin was not in the CLSI list for Staphylococcus. I recommend to delete this part "In order to evaluate the susceptibility of selected standard strains to common antibiotics (amoxicillin, penicillin, metronidazole) in the treatment of oral infections, a disk diffusion test was performed and the sensitivity and resistance to antibiotics according to the CLSI M100-standard method. S29 was determined (CLSI, 2019)" from the manuscript as it does not adding anything to the content of the manuscript.

R13: was deleted part :In order to evaluate the susceptibility of selected standard strains to common antibiotics (amoxicillin, penicillin, metronidazole) in the treatment of oral infections, a disk diffusion test was performed and the sensitivity and resistance to antibiotics according to the CLSI M100-standard method. S29 was determined (CLSI, 2019)" from the manuscript as it does not adding anything to the content of the manuscript.

Q14: Add the date of the study in methods section.

R14: line 108: Added the date of the study: This study was performed on Oct. 2021.

Q15: Add the source (manufacturer name, country) of propolis and chlorhexidine. Also, add the manufacturer name and country for all materials and devices used in the study such as TSB, TSA, TTC, and ELISA Plate Reader.

R15: lines 112-143: Added the source of Tryptic Soy Broth (TSB) (Merck, Germany) medium. Tryptic Soy Agar (TSA) (Merck, Germany) medium. To determine the MBIC of propolis aqueous extract (Roodin food industries, Iran) and CHX 0.2% (Shahre Daru, Iran), the studied bacteria were cultured on TSA medium. After overnight incubation at 37˚C, bacteria colonies were removed by loop and inoculated in 5 ml of TSB medium overnight at 37˚C. In the study of the effect of propolis aqueous extract, MBIC first using TSB medium, was prepared from each studied bacteria, a dilution equal to 0.5 McFarland separately. In each row of 96-well microplate (SPL, Korea), , the 2, 3, 5-tri-phenyl-tetrazolium chloride (TTC) (Merck, Germany) staining method was used to observe the biofilms. ELISA Plate Reader (Biotek-epoch).

Q16: The diluent for propolis is not mentioned. Also, the authors did not clarify whether the propolis was used directly or its extract was examined.

R16: Line 120. Diluent for propolis is water. propolis aqueous extract (Roodin food industries, Iran)

Results
Q17: According to the previous comment, I recommend to delete the first paragraph related to antibiotic disc diffusion.

R17: was deleted.

Q18: Move the "the percentage of inhibition of biofilm formation in each case was calculated using the following formulas" and the related formula to materials and methods.

R18: lines148-151. Moved the "the percentage of inhibition of biofilm formation in each case was calculated using the following formulas" and the related formula to materials and methods.

Q19: Write the main results such as the values of MBIC of propolis and CHX 0.2%, results of MBEC of propolis and CHX 0.2%, results of synergistic effect of propolis and CHX 0.2% on MBIC, and results of synergistic effect of propolis and CHX 0.2% on MBEC in appropriate sections.

R19: in lines 219- 256, main results was added in the text. **Results of the MBIC of** propolis aqueous extract and CHX 0.2%

The results of MBIC of CHX and propolis aqueous extract for *S. aureus* and *S. mutans* are shown in Table 1. The results showed that the MBIC of chlorhexidine for both *S. aureus* and *S. mutans* was 125 µg/ml and the MBIC of propolis aqueous extract was 937.5 and 3750 µg/ml, respectively.

Also, after measuring the adsorption rate at 490 nm, the percentage of inhibition of biofilm formation in each case was calculated it is reported in Figures 1 and 2, respectively.

Results of MBEC of propolis aqueous extract and CHX 0.2%

In this study, bacteria of *S. aureus* and *S. mutans* were used to determine the MBEC related to CHX and propolis, and the results are shown in Table 2. Also, MBEC chlorhexidine for *S. aureus* and *S. mutans* was 250 and ≥500 µg/ml and MBEC propolis 1875 and ≥7500 µg/ml, respectively.

Also, after measuring the adsorption rate at 490 nm, the percentage of biofilm remaining in each well was calculated using the formulas presented, and the percentage of biofilm eradication by CHX and propolis aqueous extract on *S. aureus* and *S. mutans* was shown in Figures 3 and 4, respectively. The lowest concentration of CHX and propolis aqueous extract, in the presence of which the absorption rate was reduced by at least 90%, was considered MBEC.

Results of synergistic effect of propolis aqueous extract **and CHX 0.2% on MBIC**

The results of the study of the synergy of CHX and propolis aqueous extract are shown in Table 3. The FBICs of CHX and propolis aqueous extract for *S. mutans* were calculated to be 15.62 and 117.18 µg/ml, respectively, and also, for *S. aureus* were calculated to be 7.81 and 58.59 µg/ml, respectively. It is postulated, therefore, that the synergistic effects shown are significant ($P < 0.05$), and MBICs in combination decreased 4- fold in comparison with MBIC alone.

Results of the synergistic effect of propolis aqueous extract **and CHX 0.2% on MBEC**

Calculating the percentage of residual biofilm removal, after the effect of CHX and propolis aqueous extract, ΣFMBEC was calculated for the tests designed in Table 4. Combining the two studied substances with 0.5 ≤ ΣFMBEC were found to have a synergistic effect. The results are shown in Table 4. The FMBECs of CHX and propolis aqueous extract for *S. mutans* were calculated to be 250 and 18.75 µg/ml, respectively, and also, for *S. aureus* were estimated to be 125 and 937.5 µg/ml, respectively. In general, the results showed that the combination of the two substances studied had an indifferent effect.

Q20: The authors claimed that they used P value of <0.05 for significant results, but these analyses were not reflected in any part of results.

R20: lines 244-246: It is postulated, therefore, that the synergistic effects shown are significant ($P < 0.05$), and MBICs in combination decreased 4- fold in comparison with MBIC alone.

Discussion

Q21: The first 4 paragraphs are not necessary and can be written as one paragraph.

R21:Lines 259-267: be written as one paragraph: Microbial biofilms are a community of microorganisms placed together by a structural matrix (31, 32). Due to the strength and structural properties of biofilms, these communities can form in different parts of the body. Dental plaque is an example of a microbial biofilm that contains layers of growing microorganisms, epithelial cells, macrophages, and leukocytes held together by an organic matrix (33). Dental plaque, like other types of biofilms, is connected through water channels and allows them to absorb nutrients and excrete waste products, this structure also creates resistance to antimicrobial agents. Dental plaque is the leading cause of periodontal disease and gingivitis, and most of all, caries, which is very important and common (34).

References

Q22: Check all references to insure that all of them are according to the journal style. Some references seem to be not correct. Also, in the main text, the references are not according to the journal style and the authors used several parentheses for each reference (such as (18)(19). While, they need to be written as follows: (18,19). Moreover, in some parts, the citation in not in appropriate order such as (13)(11) and (21)(2). Check and correct all text accordingly

R22: in lines 368- 497 was Checked all references to be according to the journal style (Vancouver style)

Tables
Q23: All tables can be designed as one table. I recommend to write the results in the text and brief the tables.

R23: in lines 229- 246, main results was added in the text.

Reviewer 2:

The current article is about Evaluation of the inhibitory synergistic effect of propolis and
chlorhexidine on biofilm cells of oral pathogens in vitro.

The comments are as follows:

Q1: This manuscript need grammatical copy editing because several errors are seen through the text.

R1: The whole text was grammatically re-examined and the deficiencies were eliminated.

Q2: In the methods of abstract, the authors need to clarify the source of studied bacteria.

R2: In line 13, was added the source of the bacteria.

Q3: The result of the abstract is so brief and no any value of the studied variables are presented.

R3: In lines 16 to 21, was added value of the studied variables.

Q4: In the introduction of main text, the objectives part is missing and needs to be added.

R4: In lines 29 and lines 99 to 105, the objective word and the aims of the study were added and specified, respectively.

Q5: The material and methods is not fully clear and need more work. It can be written in more brief but understandable style.

R5: In the Methods section, a review was performed and items were highlighted in the original text. (Lines 106- 216)

Q6: The results of main variables were not written in the result part of manuscript and the authors refer to tables only. The main results need to be added in the text.

R6: in lines 229- 246, main results was added in the text.

Q7: Check all references to be according to the journal style.

R7: in lines 368- 497 was Checked all references to be according to the journal style (Vancouver style)

2 Revision 1

1 comment · 24 Jan 2022

Editor-in-Chief

MINOR REVISION 24 Jan 2022

Dear Dr. Hadis Tavafi
Your manuscript entitled " In vitro evaluation of the synergistic inhibitory effect of propolis and chlorhexidine on biofilm cells of oral pathogens" needs more revision.

Best Regards
EIC, Dr. Mard

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Revision 2

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Reviewer 1:
Q1: The discussion did not revised according to the previous comment. Delete and brief 3 primary paragraphs (from Microbial biofilms are a community ..... till show significant antibacterial behavior) as one paragraph.

R1: Lines 259-269: 3 primary paragraphs of the discussion, Was Deleted and summarized:

Microbial biofilms are a community of microorganisms placed together by a structural matrix (31, 32). Dental plaque is an example of a microbial biofilm that contains layers of growing microorganisms, epithelial cells, macrophages, and leukocytes held together by an organic matrix (33). Dental plaque is the leading cause of periodontal disease and gingivitis, and most of all, caries, which is very important and common (34). Among the important microorganisms in forming oral biofilms can be *S. mutans*, *S. aureus*, *Lactobacillus* spp. *E. faecalis* and *Candida* species were mentioned (33). There are several mechanisms for controlling biofilm formation: 1: preventing the formation and growth, 2: destroying the biofilm structure, and 3: killing the bacteria that live in the biofilm structure. Among these mechanisms, prevention of biofilm formation and growth and colonization using antibacterial agents is the most effective method (35).

Q2: The manuscript needs a grammatical correction by a native copy editing service.
R1: The manuscript was reviewed and edited by 2 experts in the field of microbiology.

3 Revision 2

1 comment · 6 Feb 2022

Editor-in-Chief

ACCEPT 6 Feb 2022

Accept