Author's response to reviews

**Title:** Protective effect of topical Cordia verbenacea in a rat periodontitis model: immune-inflammatory, antibacterial and morphometric assays.

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**Author's response to reviews:** see over
Dear Editor,

I hope this letter finds you well.

With regard to the manuscript entitled “Protective effect of topical *Cordia verbenacea* in a rat periodontitis model: immune-inflammatory, antibacterial and morphometric assays”, it was revised conforms to the journal style, as recommended. Please find our responses to the reviewers’ comments as follow:

**Reviewer 1:**

The manuscript, “Protective effect of topical *Cordia verbenacea* in a rat periodontitis model: immune-inflammatory, antibacterial and morphometric assays.”, is acceptable for publication, however, it would need to be revised and improved before acceptance. I have attached below the comments for your use in understanding this decision. I also hope these comments will be useful in future studies.

This manuscript describes a experimental study where wistar rats were submitted for EPD for 11 days animals received 5mg/Kg of essential oils isolated from *C. verbenacea*. The therapies were administered topically 3 times daily for 11 days. Overall, the study is clearly written, but suffers from what must be described as a "Major flaw". Without a positive and negative control drug the study was designed to asses the efficacy of cordia essential oils however is not quite clear the anti-inflammatory activity without control groups. FORMAT REVIEW- STUDY DESIGN: Please compare with doxicicline and saline groups. I regret having to send you a temporally negative decision, as I can appreciate the time and effort they have expended in carrying out the study. We welcome the opportunity to review material in the future.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, and I have assessed the statistics in my report.

We thank this reviewer very much for bringing all these aspects up. In fact, the present study did not include therapeutic groups to evaluate saline and doxicicline (or other anti-inflammatory agent) effects, since the primary aim of this investigation was to
assesses the effects of *C. verbenacea* topically administered in a rat periodontitis model. As this study was the first to analyze whether the topical application of *C. verbenacea* could promote some protective effect on the periodontal destruction, only a control group using vehicle substance was used to comparisons with *C. verbenacea* essential oil group. Although it may be considered a limitation of the present trial, we believe that the absence of a saline or doxicicline group not do invalid the outcomes obtained in the present investigation, since a control group using placebo solution (vehicle) was used to demonstrate the impact of applying *C. verbenacea*. Nevertheless, we agree with this reviewer that a comparison with a saline and doxicicline group could bring additional information concerning the real relevance of *C. verbenacea* in modulating periodontitis. Certainly, this reviewer suggestion will be considered in our future investigations. The *Discussion section* was improved highlighting this aspect and emphasizing caution when interpreting the outcomes obtained in the present study.
Reviewer 2:

Reviewer's report:
The manuscript reports the protective effect of essential oil of Cordia verbenacea a rat periodontitis model, based on the results, this paper represents a contribution to knowledge in the search for natural substances to treat various alterations in the oral cavity as inflammation and bacterial infection. Suggest only consider the corrections that have been identified in the manuscript (attachment: 1858588073594847_article revised) and then mention the most important: I suggest the following keywords: Cordia Verbenaceae, essential oil, periodontitis, alveolar bone loss. anti-inflammatory, antibacterial.
This recommendation was considered, as suggested by the reviewer.

Methodology should be organized and suggests the following order:

METHODS
Plant material and extraction of essential oil
Rat periodontitis model
The animal cohort was composed of 36 male Wistar rats weighing 308 ± 35 g at the..
In order to induce experimental periodontitis, one of the…………
Treatment
Measurement of alveolar bone loss
ELISA assay
Microbiological assessment
Statistical Analyses
Thank you for this relevant comment. As recommended, the methodology was organized following the cited order.

RESULTS
Microbiological outcomes: These results should be supported by the electrophoresis of PCR products of the detection of different periodontopathogens investigated.
As suggested, a representative electrophoresis of PCR products of the detection of different periodontopathogens was added in the Figure 1.

REFERENCES
Complete references in relation to the authors.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests
Reviewer 3:

Reviewer's report:
Research article

1. Is the question posed by the authors well defined? Yes, this is a well-designed study, with clear future clinical applications. 2. Are the methods appropriate and well described? The methods are rather well-described; however, I recommend to authors to include the number of animals per group.

The number of animals per group was cited in the Treatment Item (Methods Section), as follows: “After ligation placement, animals were randomly assigned to one of the following groups, according to a computer-generated code: Non-treatment group (NT) (n=18) animals received topically 1 mL of vehicle and C. verbenacea essential oil group (C.v.) (n=18) animals received topically 5 mg/Kg body wt. of essential oils isolated from C. verbenacea.”

3. Are the data sound? Data is generally good and interesting. I would like to know why have the authors decided to measure IL-1alpha instead of IL-1beta, which is one of the most relevant pro-inflammatory cytokines. Furthermore, the authors are required to inform the rational for choosing eleven days as the interval of treatment. Finally, the authors are recommended to include the histological analysis in the manuscript.

Thank you for these relevant comments. Firstly, the decision to evaluate the levels of IL1-alpha over IL1-betawas determined based on the commercially available ELISA kits (QuantiKine; R&D Systems Inc., MN, USA). To evaluation of tissues from rats, animal model used in the present study, only ELISA kits for IL1-alpha are commercially available, and not for IL1-beta. Investigations have indicated that there are two principal forms of IL-1 that have agonist activity, IL-1α and IL-1β, with a third ligand, IL-1 receptor antagonist (IL-1ra) that functions as a competitive inhibitor. It has been described that IL-1α and IL-1β typically have similar activities (Graves & Cochran 2003: Graves DT, Cochran D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol. 2003;74:391-40; Tatakis & Kumar, 2005: Tatakis DN, Kumar PS. Etiology and pathogenesis of periodontal diseases. Dent Clin North Am. 2005;49:491-516).
Thus, the contributions of both IL-1α and IL-1β (two distinct but related molecules) to alveolar bone loss and periodontal disease have received considerable attention in the literature. In general, IL-1β expression was elevated in gingival crevicular fluid at sites of recent bone and attachment loss in patients with periodontitis (Lee et al. 1995: Lee HJ, Kang IK, Chung CP, Choi SM. The subgingival microflora and gingival crevicular fluid cytokines in refractory periodontitis. J Clin Periodontol 1995;22:885-890).

The exogenous application of recombinant human IL-1β in a rat ligature model accelerated alveolar bone destruction and inflammation (Koide et al. 1995: Koide M, Suda S, Saitoh S, et al. In vivo administration of IL-1 beta accelerates silk ligature-induced alveolar bone resorption in rats. J Oral Pathol Med 1995;24:420-434). Moreover, transgenic mice overexpressing IL-1α in gingival epithelium developed a syndrome that paralleled all of the classic features of periodontal disease, including loss of attachment and destruction of alveolar bone (Dayan et al. 2004: Dayan S, Stashenko P, Niederman R, Kupper TS. Oral epithelial overexpression of IL-1alpha causes periodontal disease. J Dent Res 2004;83:786-790). Taken together, these studies strongly point out the role of both IL-1α and IL-1β in promoting destruction of the periodontium, supporting the evaluation of the present investigation in determining the levels of IL-1α in the gingival tissue of rats by Elisa assay.

Regarding the histological analysis, it was not included in the current investigation since a morphometric evaluation was performed in order to measure alveolar bone loss. Evidences have ever showed that no significant differences were found between the two methods (morphometric and histologic analysis) in the detection of bone height associated with the placement of ligatures in rats (Fernandes et al. 2007: Fernandes MI, Gaio EJ, Oppermann RV, Rados PV, Rosing CK. Comparison of histometric and morphometric analyses of bone height in ligature-induced periodontitis in rats. Braz Oral Res. 2007 Jul-Sep;21(3):216-21), supporting the methodology employed in the present study.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes, data are appropriately described. One might suggest to present data as the mean accompanied by SEM instead of the SD, as it is currently presented.

As suggested, SD was changed to SEM.

5. Are the discussion and conclusions well balanced and adequately supported by the data? Yes, discussion section is satisfactory and the authors cite the appropriated references. The authors should mention the existence of a commercial preparation containing C. verbenacea for treating musculoskeletal pain.

This recommendation was considered, as suggested by the reviewer. A paragraph mentioning this aspect was added in the Discussion Section. “Recently, the Brazilian market released this plant for the industrial production of therapeutic agents, and is available a commercial preparation with C. Verbenacea to treat musculo-skeletal disorders and tendinitis.”

6. Are limitations of the work clearly stated? Yes.

7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes. This is correctly presented by the authors.

8. Do the title and abstract accurately convey what has been found? The abstract and the title are adequate.

9. Is the writing acceptable? Yes.

As a general evaluation, I recommend the acceptance of the manuscript, with minor revisions, as cited in the items before.
Reviewer 4:

Reviewer's report:

Dear Authors, this manuscript describes the effects of topical Cordia Verbenacea in a rat periodontitis model evaluating its anti-phlogistic and antimicrobial activity by immune-inflammatory, antibacterial and morphometric assay. The rationale for doing the study is reasonable, representing, at our knowledge, the first report on the effect of C. Verbenacea on induced periodontitis in a rat model. The methods are appropriate and well explained. The authors show good and clear description of the assays used as well as the parameters investigated. The results are clearly presented and the discussion is generally relevant to the current literature.

Major Compulsory Revisions

1) Materials and methods. Regarding the paragraph “Plant material and extraction of essential oil”: additional data about the Cordia Verbenacea's chemical composition used in the experiment are required.

2) Results: A table containing the results of the compounds identified by the GC-MS analysis of C. Verbenacea is suggested. Only the role of alpha humulene is analyzed. Since several works cite the role played from other active components contained in C. Verbenacea, the Authors are asked to explain why other components are not considered. Moreover, any substances involved in the antimicrobial role are indicated or speculated.

We thank the reviewer for bringing this topic up. The reason for the present study has focused on alpha humulene evaluation was based on previous scientific evidences that support this decision. According to Passos et al. 2007 (Giselle F. Passos, Elizabeth S. Fernandes, Fernanda M. da Cunha, Juliano Ferreira, Luiz F. Pianowski, Maria M. Campos, João B. Calixto; Journal of Ethnopharmacology; Volume 110, Issue 2, 21 March 2007, Pages 323–333) alpha-humulene was the major compound identified as involved with anti-inflammatory activity, therefore this study employed this compound as active principle for quality control purposes. This data was also corroborated by Medeiros et al (2007) that showed that α-Humulene and trans-caryophyllene are sesquiterpene compounds identified in the essential oil of Cordia verbenacea which display topical and systemic anti-inflammatory effects in different experimental models (Medeiros, R. Giselle F. Passos, Vitor, C.E., Koeppe, J., Mazzucco, T.L., Luiz F. Pianowski, Maria M. Campos João B. Calixto; British Journal of Pharmacology;
Michielin et al. (2009) also described that alpha-humulene and trans-caryophyllene are the main compounds responsible for the anti-inflammatory and anti-allergic characteristics attributed to the medicinal plant Cordia verbenacea D.C., hence giving importance to use as parameters for quality control of the essential oil. (Eliane M.Z. Michielin, Sibele R. Rosso, Elton Franceschi, Gustavo R. Borges, Marcos L. Corazza, J. Vladimir Oliveira, Sandra R.S. Ferreira; The Journal of Chemical Thermodynamics, Volume 41, Issue 1, January 2009, Pages 130–137).

The main focus of this study was to evaluate the effect of crude extract and essential oil of Cordia verbenacea (C.V.), systemically administered on ligature-induced periodontitis in rats. Therefore the strategy applied in this study was to employ the parameter already established as validated for quality control purposes.

3) Discussion: The role of specific components of C. Verbenacea with a supposed antiphlogistic and/or antimicrobial activity needs to be better explained. The issue “Accordantly, Michielin et al. [33] revealed that some extracts from C. Verbenacea were effective against both Gram-positive (Staphylococcus aureus and Bacillus cereus) and Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa)” has to be evaluated. In fact the cited authors clearly differentiate the antimicrobial role of C. Verbenacea on gram positive bacteria against a limited role exerted on gram negatives.

We thank this reviewer very much for bringing this aspect up. In fact, Michielin et al. (2009) revealed that some extracts from C. Verbenacea were more effective against Gram-positive than Gram-negative bacteria. The paragraph in Discussion section was amended and aspects concerning the antibacterial action of C. Verbenacea were clarified.

4) Statistics: Could the Authors clarify the meaning of a so wide Standard Deviation values in IL1 and IL10 results obtained with ELISA immuno assay?

Thank you for this observation. The standard deviation values obtained in ELISA assay in the present study represent an individual variability and it may be frequently observed in methods of chemical assay. Other studies using different experimental models also demonstrated that the pattern of cytokine profiles obtained by enzyme-

Minor essential revisions

Abstract: For an easier comprehension the issue “Animals were randomly divided into two groups: non-treatment group (NT)(n=18): animals received 1mL of vehicle or C. verbenacea group (C.v.)(n=18): animals received 5mg/Kg of essential oils isolated from C. verbenacea” could be corrected in “a) non-treatment group (NT)(n=18): animals received 1mL of vehicle; b) C. verbenacea group (C.v.)(n=18): animals received 5mg/Kg of essential oils isolated from C. verbenacea”.

Thank you for this important suggestion. The recommendation was accepted and the modification was performed.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: 'I hereby declare that I have no competing interests.

Thank you very much for contributions and suggestion recommended.

Sincerely,

Fernanda V. Ribeiro (Corresponding Author)