Title: Topical Green Propolis Improves Corneal Wound Healing and Inflammation in Rats following Alkaline Burns

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Author's response to reviews: see over
To Tom Rowles
Executive Editor
BMC Complementary and Alternative Medicine

Ribeirão Preto, August 16th, 2013.

Dear editor,

Enclosed is a copy of our revised manuscript entitled “Topical Green Propolis Improves Corneal Wound Healing and Inflammation in Rats following Alkaline Burns”, which we would like to submit for publication in BMC Complementary and Alternative Medicine as an Research Article.

The comments of the reviewers really enhance the manuscript quality. So, a revision was carefully done according to the reviewers’ comments. All manuscript changes were also indicated in the attachments.

All authors have approved and are fully conversant with its contents and also have agreed to the modifications made in this article. We declare that there are neither actual nor potential personal or financial interests involved in the material, information or techniques used in this paper.

Thank you for your kind attention. We hope to hear from you soon.

Sincerely Yours,

Jayter Silva Paula

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Reviewer 1: Andresa Berretta

Reviewer’s report:

The authors would like to thank the reviewer Dr. Andresa Berretta for her careful reading and key suggestions that certainly improved clarity and accuracy of our manuscript. The changes were presented in the text using the “track changes” feature of Word processor and commented in the following pages. All the authors read and agree with the suggestion made based on her suggestions.

Major Revisions are required.

The manuscript of Martin et al. presented like the aim of evaluate the antiinflammatory and healing actions of a green propolis microemulsion in alkaline injuries in eyes.

Some points need to be clarified, especially considering to propolis source, extraction and pharmaceutical preparation, for example:

1. The authors inform that Green propolis used in the work was obtained from Barra do Corda, MA, Brazil. However, green propolis is a very specific type of Brazilian propolis and is associated with southern and part of south region of this country, especially Minas Gerais and São Paulo States, besides this propolis is characterized for presenting Artepillin C like an important biomarker from source. Considering that green propolis has been extensively studied nowadays, and the source mentioned in the present manuscript looks like strange, is mandatory that the authors present chromatographic fingerprint of the propolis used in order to confirm the denomination used in the work, if this chemical characterization was not possible, I can strongly suggest that the authors change the denomination used for propolis in this manuscript because, until now, scientific literature do not presented demonstration of green propolis in Northeast region of Brazil.

ANSWER: We agree with the reviewer that Green Propolis is not the appropriate name and change it to Brazilian propolis in the whole text based on the following arguments: 1) We used propolis extracted from samples of the native Brazilian stingless bee colonies (Scaptotrigona sp) in Barra do Corda, Maranhão state; 2) Stingless bees are found in many tropical regions and are particularly known as the major native pollinators of flowering plants in the tropics, including Brazil; 3) These bees are found in Maranhão, a Northeast Brazilian state mentioned as the local of sample source; 4) The recently performed chromatographic analysis of extract of Scaptotrigona sp propolis showed differences if compared with those of green propolis. This study was presented in a Brazilian congress, however it was not published yet [Santos et al., 2012. Análise dos constituintes...](#)
The authors are using propolis produced by Scaptotrigona sp., one type of propolis very little studied. However, the introduction and discussion does not mention the reasons for this choice, and more impressive is to compare the results with other types of propolis, especially the concentration with red propolis, a completely different type of propolis. Then, I suggest that the discussion of the results involves propolis from Scaptotrigona sp., or, if not available because the limited number of studies with this specific type of propolis, use results obtained with brown or green propolis, that represents more properly the general chemical composition of propolis, and not red propolis;

**ANSWER:**

a) We chose propolis from Scaptotrigona sp because these bees are stingless, eusocial and less harmful to humans, making the samples of propolis more easily obtained. Therefore, it may represent a Brazilian alternative to red and green propolis, not yet published as an ocular modulator of inflammation and healing. A phrase emphasizing this point was included in the Background section (pages 2, 3);
b) Regarding the comparison with other propolis, we agree that displaying findings related to both green and red propolis would be of interest for readers. Please consider modifications on pages 2, 9 and 10.

3. Was microemulsion prepared with raw propolis crushed or some extract of raw propolis was used? In this last case, what kind of extractor solvent was used, process, temperature, etc.? Could microemulsion preparation be better explained?

ANSWER: Microemulsion was prepared with raw propolis crushed. It was prepared by solubilization of the raw extract in the concentration of 1.0% with particles of 30nm in a mixture compound by polyethylene glycol-6- caprylate / caprate, polyglyceryl-6-dioleate, glycerides caprylate / caprate (10.0%) (MACKADERM MicroExpress - McIntyre, USA), chloride benzalcone (0.01%) and water distilled deionized 100% per system MilliQ (Millipore, USA). These details of microemulsion preparation were added to the text, Methods section, page 6 and 7.

4. The authors inform that control group was treated with vehicle, can the authors clarify what vehicle involves.

ANSWER: Yes, the vehicle used involved polyethylene glycol-6- caprylate / caprate, polyglyceryl-6-dioleate, glycerides caprylate / caprate (10.0%) (MACKADERM MicroExpress - McIntyre, USA), chloride benzalcone (0.01%) and water distilled deionized 100% per system MilliQ (Millipore, USA). This information was also included in the Methods section (page 6).

5. The quality of the work can be improved if a group treated with a conventional medicine is introduced, like a group treated with a corticosteroid, for example.

ANSWER: We agree that a “positive control” group could enhance the discussion in some points. However, an undoubted healing corneal agent (gold standard) is neither well described nor available for using in the clinical practice. Moreover, in the present work our aim was to study whether Brazilian propolis had effects on corneal inflammation, but mainly on the wound healing. Testing other anti-inflammatory drugs like corticosteroids would also add new vehicles and distinct stabilizing compounds, which may counteract the healing process with anti-inflammatory effects. The considerations on this issue were added on page 11.

6. Because some strange word can be found in the text, the revision of the language is indicated, for example: “principally”, “a resinous product consisting of sap, bark and be
excreta”, etc. The work is important to propolis field and can contribute to the knowledge in this area.

ANSWER: Thanks for all your encouraging corrections and comments. We have also performed a new grammar revision with a native English speaker, Prof Dr. Peter Reinach, visiting professor at our institution and emeritus professor of State University of New York.
Reviewer 2: Luciola Barcelos

The authors would like to thank the reviewer Dr. Luciola Barcelos for her detailed comments and important suggestions that certainly improved the clarity and accuracy of our manuscript. The changes were presented in the text using the “track changes” feature of Word processor and commented in the following pages. All the authors read and agree with the suggestion made based on her suggestions.

Reviewer’s report:

Major Compulsory Revisions:

1. It was already known that topical propolis has an anti-inflammatory effect on alkali-injured eyes (The Effect of Propolis Extract in Experimental Chemical Corneal Injury. Öztürk F et al. Ophthalmic Res 2000;32:13–18 (DOI: 10.1159/000055581)) and authors must not only reference this work but also discuss the differences (if any) between their finds and/or model used and that one that could characterize novelty in this aspect of their work.

   ANSWER: We agree that the study “The Effect of Propolis Extract in Experimental Chemical Corneal Injury”, produced by Öztürk F et al. is an important work regarding their contribution on the description of the effects of a propolis extract in corneal wound healing and inflammation. Nonetheless, some differences should be discussed. First of all, they used a different type of propolis, red propolis. Also, in their study the alkali burn was induced by applying a filter paper immersed in NaOH 1N in rabbits. In a pilot experiment performed before our presented study, we performed lesions using the same protocol of injury, but the limits of the injured area were repeatedly very imprecise. That is a very important issue and probably was the main reason that led them not to study the healing process of the wounded area.

   The inflammation evaluation was another relevant point to discuss. Öztürk F et al. performed clinical evaluation, using observation of the conjunctival hyperemia, and corneal edema, with no specific quantitative analysis. Therefore, we developed a model in which the animals were examined and photographed in an ophthalmic slit lamp, allowing a more accurate evaluation. We added comments regarding the results of this study in the Discussion section, including also its reference. Please consider pages 2 and 10.

2. Regarding the inflammation analysis, authors should give detailed information about the parameters that were used to identify the leukocyte infiltrate as well as a more accurate explanation if they performed total and/or differential counts of leukocytes. None of those
information is stated anywhere. In fact, in the Material and Methods section they claim they count “leukocytes” (it’s not clear if total or not) and, in the Table 2, the data is presented as “neutrophil” count.

ANSWER: The authors agree with this point and rephrased it throughout the paper to emphasize that our observations were limited to counting neutrophils infiltrate in the cornea. Besides, due to the issues related to our description of cell migration in the cornea and the rationale presented in your comments (#2 and #3), we also optioned to exclude the differential analysis of cell infiltration in the center and borders of cornea, as requested. Please consider the modifications on page 6.

3. Regarding leukocyte migration analysis in situ, I’m not convinced about the accuracy of the methodology the authors have used. If this is a standard methodology to access this parameter on corneal injuries, authors should give a reference for this method. Considering that there is no newly formed vessels at the time points evaluated and that the cornea is an avascular structure and so that leukocytes should come from vessels from other regions than cornea at those time points, the leukocytes present in the borders, but not necessarily in the center of the wound, would already represent cells that have been migrating. In addition, it’s not clear why authors count leukocyte migration (that I’m considering as total leukocyte count) as “center/total ratio” and subsequently they consider count neutrophils “center/border ratio” as a second parameter of leukocyte migration. Which is the difference between those “parameters”? Also the description of this analysis is very confuse and should be revised.

ANSWER: We also agree. Considering the issues pointed and discussed in the comment 2, we withdrew these evaluations, and keep presenting only the total amount of neutrophils of the groups.

4. As propolis chemical composition, that influences its activity, depends not only on the phytogeographic characteristics of the place of collection, but also on the solvent used during the extraction process and the information about which solvent was used during the extraction is not stated anywhere in their work, authors should describe it on Material and Methods section.
ANSWER: We agree with the reviewer comment and included the information in the Methods section (page 5). The extract used in the present work was obtained directly from beehives using water and ethanol 70% (7:3).

5. Still regarding propolis chemical composition, what are the most bioactive compounds present in the extract used in the present work? There is no analytical data of the components of the extract used in the present work or cited reference for that. This information should be given.

ANSWER: We also agree that analytical data of the compounds used could add some information. In this study, we did not perform analysis of the components of the extract, but some previous studies detailed that information:


We are aware of the importance of knowing which components are the main responsible for propolis effects and that the extracts may have some variation depending not only on the conditions of location, weather and vegetation, but also on the month of the year they were collected and conditions of extraction itself. Therefore, some minor variations may exist between our extract and the extracts used in the articles cited, but standardization of extracts is a problem in almost every study published using propolis extracts. The focus of the study was to find out weather Brazilian propolis had some effect in corneal wound healing and inflammation, as it is the first study using a propolis other than red in the cornea. Considering those studies, we commented some of these limited data in the discussion section, on page 11.

Minor Essential Revisions:

1. Authors should consider display representative images of fluorescein-stained eyes and representative H&E images of histological sections of corneas after injury for illustrating their finds on differences between green propolis and vehicle regarding wound closure and inflammation, respectively.
ANSWER: We have included the images of fluorescein-stained injury corneas at representative time points, as well as H&E image of a histological section, displaying inflammation and the necrotic area (Figure 1). We believe that data presented in the tables, regarding the comparison of wound healing and inflammation between groups, are highly explicative and it would no longer be necessary to show all images.

2. If authors choose for showing differential leukocyte count (instead of total leukocyte counts) at wound sites, they should be more accurate in the naming of cells as polymorphonuclear and mononuclear leukocytes as any specific analysis (e.g. immunohistochemistry using specific antibodies) was done to state which cell type was exactly present at wound sites.
   ANSWER: As mentioned above (comment 2), we have performed a neutrophil (polymorphonuclear cells) count, which could be done using a light microscope by an experienced pathologist. Please consider those modifications.

3. Authors should state in the Material and Methods section how many drops per eye per application and what was the volume of each drop used in their experiments.
   ANSWER: We agree. Two drops of 40uL were applied, 10 seconds apart per application. Please see this description added to the text, on page 6.

4. Considering experimental animal ethics, authors should change the word “sacrificed” for “euthanized” everywhere.
   ANSWER: That’s correct. We have changed the words throughout the text, as suggested.

5. The description of “wounded area” result should be better elaborated as that reader could have a clear notion that propolis reduces the area of wounds.
   ANSWER: We also agree. Please consider the modifications in the phrase construction on pages 1 (Abstract) and 8 (Results).
Reviewer 3: Alison M McDermott

The authors would like to thank the reviewer Dr. Alison M McDermott for her important comments and corrections. All the suggestions certainly improved the clarity and accuracy of our manuscript. The changes were presented in the text using the “track changes” feature of Word processor and commented in the following pages. All the authors read and agree with the suggestion made based on her suggestions.

Reviewer's report:

Major Compulsory Revisions

1. The description of how leukocytes were counted is not clear. How was the center of the lesion determined? Why were intermediate fields not counted? I do not understand the phrase “...with posterior calculation of the number of cells.”

   ANSWER: We agree with the reviewer. We chose to evaluate the neutrophils amount instead of differential leukocytes counting because our primary interest was to evaluate the acute phase of inflammation associated with the corneal injury. So, we modified the description, using only “neutrophils” nomination throughout the text. Besides, due to the difficult of meaning and problems related with the description of cell migration, we optioned to exclude that analysis. We also withdrew those evaluations, and keep presenting only the total amount of neutrophils. These cells should come from the peripheral vessels and their total amount could be considered as a good parameter of cell infiltration. Please consider the modifications on page 6.

2. The immunohistochemical analysis for Ki67 staining is not quantitative. For it to be quantitative the signal would have to be related to the amount of Ki67 present. The number of Ki67 cells can be counted and reported as has been done but the term quantitative cannot be used to describe the analysis.

   ANSWER: Thanks for this important remark. Please consider this correction on page 6.

3. Plotting the wound healing data in graphical form (e.g. % healing vs time) and including images of the fluorescein staining for vehicle and treated rats would be a more informative way of presenting the data.

   ANSWER: A composited image of corneas of representative time points is now presented (figure 1 modified). Taking the results of table 1 and the new figure 1, we believe that readers could have the exact idea of the dynamic healing process observed. Thanks for your suggestion.
4. I do not understand the comment about leukocytes being present in “all extensions of cornea stroma”. What is an extension of the corneal stroma? Also it is quite clear that the majority of the inflammatory cells are confined to the anterior stroma which should be noted. The area of central necrosis in epithelium and stroma should be identified in Fig 1.

ANSWER: We also agree. We may explain that our first intention was to refer to the presence of inflammatory cells in the central, lateral (peripheral) and intermediate stroma areas. However, we realized that this term was unclear in the text. The correction was made on page 8, taking your suggestion in account. We also identified the area of central necrosis in the modified Figure 1 (E and F).

5. Where are the counts in Tab 2 from? Sum of all three counting areas?

ANSWER: Yes, the counts in Tab 2 represent the sum of all three counting areas. We added this information in the Methods section, on page 6.

6. The specificity of the Ki67 staining is questionable. The corneal endothelium, which divides infrequently, is stained positive for Ki67 suggesting some artificial staining. A background control must be included.

ANSWER: This is an important point to debate. Firstly, positive stained for Ki67 could be considered a good and specific evidence of cycling cells in several tissues. In fact, labeling of cycling corneal endothelial cells with Ki67 was demonstrated in cats during the healing process some years ago (Petrol et al. Labeling of cycling corneal endothelial cells during healing with a monoclonal antibody to the Ki67 antigen. Cornea 1999; 18(1):98-108). Moreover, endothelial repair of injured corneas involves endothelial cells proliferation, as proved also in rats through specular microscopy, histological staining and autoradiographic analysis of incorporation of tritiated thymidine in the nucleus (Tuft et al. Endothelial repair in the rat cornea. Invest Ophthalmol Vis Sci 1986:27(8): 1199-204). We believe that some proliferative activity of the endothelium could be presented in this burning model, however it is hard to prove with our results and was not our primary interest. Moreover, the pathologist involved in this study does not routinely take weak stained cells in account due to the possibility of non-immunological binding of substrate reaction products. As all our slices, including those presented in the figure 2, did not show strong staining of endothelial cells, we optioned to keep this presentation.
7. The comment in the discussion about counts in the VH group being 50% higher than GP group needs to be revisited. 449 is not 50% more than 355.

ANSWER: Thanks for this important observation. The percentage should be 25% instead of 50% and the correction was made in page 11.

Minor essential revisions

While very well written overall there are many instances where the English can be improved.

ANSWER: Thanks for all your encouraging corrections and comments. We have also performed a new grammar revision with a native English speaker, Prof Dr. Peter Reinach, visiting professor at our institution and emeritus professor of State University of New York.