**Author’s response to reviews**

**Title:** Antimicrobial and antiproliferative activity of essential oil, aqueous and ethanolic extracts of *Ocimum micranthum* Willd leaves

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Antimicrobial and antiproliferative activity of essential oil, aqueous and ethanolic extracts of *Ocimum micranthum* Willd leaves

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We send you, a point-by-point response letter that accompany us revised manuscript. The letter provide a detailed response to each reviewer, describing exactly what amendments have been made to the manuscript text and where these can be viewed.

The specific changes suggested by reviewers were highlighted in yellow color into the manuscript.
The review and changes of English idiom suggested by reviewers were highlighted in gray color into the manuscript.

Reference # 18 was added in the Methodology section (page 8 line 191) and Reference section (page 22 line 555-556). 

The answers point-by-point to each reviewer is described below.

Sincerely.

PhD. José Antonio Azamar Barrios

Cristiane Yumi Koga Ito (Reviewer 1):

GENERAL COMMENTS:

The conclusion section was restructured and rewritten

Evidence:

Page 19 line 465-475.

The English idiom of the manuscript was reviewed

INTRODUCTION:

The rationale of the study is not clear. Please, consider revising the last paragraph of this section.

The Introduction section was rewritten and new references were added.

Evidence

Background section:

• Page 4, line 79-103

• Page 5, line 104-114

Reference section:
METHODOLOGY:

1. The nomenclature of the micro-organisms should be revised, i.e., Page 5, line 13 - Gram should be written with the first letter in capital (Gram). Pseudomonas should be revised.

   The nomenclature of the micro-organism was corrected. The word Pseudomonas was written according to the ATCC.

Evidences:

- Abstract section: Page 2, paragraph 2, 3, line 35, 41
- Methods section: Page 7, paragraph 1, line 160
- Results and discussion section: Page 11, paragraph 2 line 269, Table 1. Antimicrobial activity of essential oil and extracts of Ocimum micranthum Willd leaves. Page 12, paragraph 1, line 283.

2. Page 8, line 13 - Usually, the incubation period for C. albicans in antimicrobial testing is 24 h with an additional reading after 48 h of incubation (for instance in CLSI standard methodology). Why did the authors adopt 40-42 h of incubation for antimicrobial tests with C. albicans? Why incubation time for the other species was 20-21 h?

   Although CLSI standard methodology indicates 24 h such as an incubation period with an additional reading after 48 h for C. albicans, we decided to report the results from a period of 40-42 h (double the time used in the pre-inoculum) because we wanted to avoid a false-negative in the test, since that characteristics such as metabolism, the type of cell division, and components of cell wall are not the same as the bacteria (incubation period of 20-21 h). Therefore, reporting results from an incubation period of 40-42 h was a cautionary measure. However, the plates were also revised at 20-21h, but were not observed changes in the results with respect to the incubation period of 40-42. Arikan (2007) has mentioned an incubation time of up to 48 h for C. albicans in this type of investigation.

   Concerning S. aureus, B. subtilis, P. aeruginosa, the methodology describes that in order to carry out the growth kinetics, the pre-inoculum of each microorganism was incubated for a period of 20-21 h (Methods section, Page 7, line 168-169), and this same time period was used in the MIC test. This incubation period was chosen for operative reasons within the laboratory, as there is a
restriction on access time, whereby it was adjusted between the minimum and maximum time recommended for this type of microorganism.


3. Do the authors believe that extracts also interfere on the iodonitrotetrazolium chloride assay, similarly to MTT?

The probability of interference exists since the test is based on a redox reaction between the products of metabolism (specifically respiratory chain) produced by the microorganisms and the tetrazolium salt reagent (such as iodonitrotetrazolium chloride). The presence of some compounds in the extracts that have the capacity to carry out this redox reaction (for example phenolic compounds) could also cause the characteristic change in color observed in the test. For this reason, diverse controls were applied and evaluated whilst the technique was conducted, such as the culture medium control (with and without tetrazolium indicator), culture medium control with antibiotic and indicator, culture medium control with indicator and dissolvent dimethylsulfoxide (DMSO), color control of each extract concentration (with and without indicator) and positive control of growth (with indicator), positive control of growth with antibiotic and indicator, positive control of growth with dissolvent dimethylsulfoxide (DMSO) and indicator, hydro-alcoholic solution with indicator, hydro-alcoholic solution with microorganism and indicator.

4. Considering that the inhibition was 100% for all the species, the period in the methodology "The inhibition percentage was calculated in all cases where the growth of microorganisms in the petri dish took place. This percentage was calculated using the quantity of inoculated aliquot, the result of CFU/mL in the petri dish test and the initial concentration of the microorganism (1.5 x 10^8 CFU/mL)." should be suppressed. Regarding this period, it seems that using the initial concentration as a basis for this calculation should be revised.

The reviewer suggests that the initial concentration of viable cells in the theoretical concentration (1.5 x 10^8 CFU/mL) should not be used as a basis for to do the calculation of inhibition percentage. Therefore, the paragraph was rewritten and the idea reformulated, instead we only suggest that a growth of microorganisms in the petri dish indicates a bacteriostatic effect, while no growth of the microorganism indicates a bactericide effect. Concerning Candida albicans, the terms are fungistatic or fungicide effect.
Evidence:


Results and discussion section: Page 12, paragraph 1-2, line 282-294.

5. After the incubation period, the number of cells will vary significantly. Maybe, using the number of cells of a positive control would be better.

A standard count in a petri dish of the volume of the inoculum used in the test could be a preferable means to calculate the number of viable cells in the theoretical concentration obtained by the turbidimetric method of Mc Farland (1.5 x 10^8 CFU/mL). This count could provide a more reliable inhibition percentage.

We decided to rewrite the section of Results and discussion, only reporting the complete qualitative information, as the observed effect (bactericide, bacteriostatic, fungicide, fungistatic) in the petri dishes.

Evidence:

Results and discussion section: Page 12, paragraph 1-2, line 282-294.

6. Why were healthy human breast-derived fibroblasts (hFB) and CHO-K1 cells used, instead of cancer cell lines for antiproliferative assays.

The Ocimum micranthum Willd is a plant used in traditional medicine in the area of the Yucatan peninsula in México for the treatment of cutaneous infections and wound healing amongst other applications [Sánchez-Medina et al. 2001]. These properties have been previously reported for the ethanolic and aqueous extracts of other species of the genus Ocimum such as Sanctum Linn (Shetty et al., 2008; Goel et al., 2010), but for the species micranthum previous research does not exist, therefore we decided to verify this property in its essential oil and the extracts (ethanolic and aqueous) using a model of proliferation in vitro as a first step. We needed to use an appropriate cell line model, and we chose to use healthy human fibroblast (Thakur et al., 2011) and a model of adherent cells as reference such as CHOK-K1.

REFERENCES:

The cytotoxicity evaluation of the concentrations that showed antimicrobial activity should be added to the manuscript.

Since some extract concentrations with antimicrobial activity (125, 250, 500 μl/mL) modified the osmolality of the culture medium to values outside of the optimum range (230 to 400 mOsm/kg) that has been suggested for a cell test (Goswami et al. 2011), these were not evaluated in the cytotoxicity assay by MTT method. The purpose was to determine the cytotoxic effect of the extract without a possible interference by osmotic shock which could affect the results. Each extract presented different concentrations that complied with the optimum range of osmolality. The purpose of the present study was to at least find a concentration of the extract that has both activities, antimicrobial and proliferative.

REFERENCE:


RESULTS AND DISCUSSION:

8. The results obtained for positive controls such as amikacin (4 mg/L) and nystatin (2 mg/L) should be cited.

The reviewer’s suggestion was considered, and the results obtained for positive controls were incorporated in the paper.

Evidence:

Results and discussion section: Page 11, paragraph 2, line 270-273
9. Some parts of the texts are not clear and should be revised, i.e. "The ethanolic extract presented the least MIC (5 μL/mL) for Candida albicans, followed by aqueous extract (80 μL/mL)."

This paragraph was rewritten.

Evidence:

10. The inhibition of 100% of all the strains is an interesting, but at the same time intriguing result, mainly considering B. subtilis. Please, discuss deeper this point.

In the results section, the paragraph was rewritten to consider the changes in the methodology, where we now only indicate a qualitative focus on the effect observed in petri dishes, bactericide or bacteriostatic in the case of bacteria S. aureus, B. subtilis, P. aeruginosa. In the case of C. albicans, the terms used were fungicide of fungistatic. These effects were discussed in the last section mentioned.

Evidence:
Results and discussion section: Page 12, paragraph 1-2, line 282-294.

CONCLUSION:

11. The conclusion section is too long. Please revise pointing out objectively the main conclusions.

This section was rewritten.

Evidence:
Conclusion section: Page 19, paragraph 2-3, line 465-475.

12. In the conclusion section, the information about the limitation of the use of MTT in the context of the study is interesting but should be moved to discussion section.

The information was moved to the Results and discussion section, and the conclusion section was rewritten.
Evidence:

Discussion section: Page 18, line 444-451, 452-457.

Conclusion section: Page 19, paragraph 2-3, line 465-475.
QAMAR UDDIN AHMED (Reviewer 2):

GENERAL COMMENTS:

The English idiom of the manuscript was reviewed.

1. The major drawback of this research work is that authors didn't attempt to isolate active principles.

   The Ocimum micranthum Willd is a plant used in traditional medicine in the area of Yucatan peninsula in México and other sites of Central America such as Guatemala. The people use a decoction of the flowers for treating gastrointestinal, respiratory and nervous diseases, as well as to relieve earache, headache, swelling, and constipation. The stems and leaves have been used in a process of maceration in alcohol to treat rheumatism, sprains and cramps. The decoction of the leaves of the plant has been used for stomach pain, rheumatism, gout, fever, high blood pressure, toothache, sores, ulcers, cutaneous infections, wounds, grains, insomnia, and menstrual regulation as well as a postpartum bathroom and mosquito repellent (Morataya-Morales, 2006; Sanchez-Medina et al, 2001). At present, the research about crude extracts of plants used in traditional medicine continues in the search of antimicrobial properties and evaluation of their cytotoxicity (Elisha et al. 2017).

   We decided to investigate the crude extracts of this plant for antimicrobial and proliferative activity on healthy cell lines, based on anecdotal evidence such as the therapeutic use of Ocimum micranthum Willd in traditional medicine for the treatment of cutaneous infections and wounds, and since there are no scientific reports that support these bioactivities; however, crude extracts and essential oil of other species of the genus Ocimum such as sanctum linn, gratissimum linn, kilimandscharicum have been reported to exert wound healing properties (Shetty et al, 2008; Orafidiya et al, 2003; Paschapur et al, 2009). With these tests, we attempt to offer evidence (research-based) for its bioactivity and effects on a healthy cell line associated with the process of wound healing.

   However, considering the potential of the results shown in these studies, it may be interesting to further investigate compounds that are directly involved in the bioactivities as well as the mechanism of action. The isolate of active compounds, as well as studies in vivo, are an example of the complementary tests to be carry out.

REFERENCES:


Elisha IL, Botha FS, McGaw LJ, Eloff JN. The antibacterial activity of extracts of nine plant species with good activity against Escherichia coli against five other bacteria and cytotoxicity of extracts. BMC Complement Alternat Med. 2017; 17:133


2. Introduction is weak and lacks important information about this plant. Traditional claims of this plant have not been documented and discussed properly. Data is not enough.

We considered the observations of the reviewer, and we rewrote the Introduction section.

Evidence

• Page 4, line 79-103

• Page 5, line 104-114

3. Authors should have tried to discover active principles responsible for aforesaid biological activities which they didn't attempt to isolate. Surprisingly, polar extracts showed positive activities in all the biological assays taken into consideration, yet no attempt was made to isolate active principles. Authors have not cleared in this paper why they were not prompted to isolate biologically active compounds from the same plant despite the fact that extracts displayed potent activities in different biological assays?

The initial purpose of this research was to evaluate the crude extracts because of the way in which the people use this plant in Traditional Medicine with pure empirical knowledge of the
functional properties and we attempt provide here a scientific explanation for the proliferative activity and antimicrobial activity. The results obtained in the present study indicate that further research will be necessary to investigate the compounds directly involved in the functional properties, as well as the mechanism of action; reasoning that justifies the isolation of active compounds and other tests.

Concerning the antimicrobial effect, some studies have reported that this activity in crude extracts may present as a synergic effect of its diverse compounds against microorganisms rather than the sole effect of the main compound. This synergic effect has been observed when volatile compounds are responsible for the antimicrobial activity.