Author's response to reviews

Title: Petiveria alliacea extracts uses multiple mechanisms to inhibit growth of human and mouse tumoral cells.

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Version: 3 Date: 5 August 2008

Author's response to reviews: see over
Bogotá, August 5 2008

Dear Sirs

BMC Complementary and Alternative Medicine

MS:1587267944203683

Article Title: Petiveria alliacea extracts uses multiple mechanisms to inhibit growth of human and mouse tumoral cells.

We have address all the comments suggested by the reviewers point by point. Attaining your recommendation a Native American English speaker colleague has copyedit the paper for language revisions. Next you will find the explanations for each reviewer.

Answers to the reviewer’s commentaries

Reviewer: LUCIA Cavallaro

- Major Compulsory Revisions

1) The authors have been to revise the references corresponding at his sentence in the section Introduction: “South American countries, alcohol and water infusions had been used for leukemia and breast cancer patients with reportedly good efficacy and a general lack of toxicity (11 12)”.

- New references describing the ethnopharmacology and toxicity of Petiveria alliacea replace references 11 and 12.

2) In the section Methods,

   a) Cell lines and growth conditions: Not figure in this section the human normal fibroblast. I’m consider that cell line have been included.

- The protocol used for fibroblast culture was included under methodology.

   b) Cytotoxicity assay. This subtitle have been reorganized between cytotoxicity assay and Normal cells assays.

- The figures names were change as suggested and the cytotoxicity assay organized.

   c) LD50 is used for the cytotoxic activity assays in vivo (i.e. animal model). IC50 (inhibitory concentration 50) is the correct denomination for the cytotoxic activity in vitro assays. I’m suggest the inclusion of the curve doses-response for each cell lines and the determination of these corresponding IC50 values.
• The IC50 for F4 fraction was calculated for each cell line treated and plotted into a new graph. Dose-response curves for all tumor cell lines and normal cells are shown.

3) The authors have been indicated for each assay, the cell lines utilized.

• At each assay the type of tumor cell line or normal cell used for the experiment is clearly specified.

Results

a) Figure 2. Title suggested: Induction of morphological changes in tumor cells by F4 from Petiveria alliacea

• The figures names were change as suggested

b) Figure 2D. Effect of F4 Pa on the depolarization of mitochondrial membranes on the Erytroleukemia cell line (mouse?). The authors should be indicated in other figure this activity.

• A new figure was added showing F4 fraction effect over mitochondrial membrane depolarization, stating K562 as a human tumor cell line.

c) Figure 3. Effect of F4 against normal cells (Human normal fibroblasts and PBMC) These curves doses-response have been included concentrations more high that 125 µg/ml. It is strongly recommended that the authors do a complete concentration-response study for to determinate the IC50 for each cell type. Similar consideration for the inclusion of the curves dose-response for tumoral cell lines evaluated in this work.

• The highest concentration used to evaluate F4 fraction treatment was 125 µg/ml in tumor cell lines and normal cells. Is worthwhile to evaluate a plant extract in vivo if the activity is under 50 µg/ml. In figure 2 A to F, number 1 corresponds to 125; number 2 to 62.5; number 3 to 31.2; number 4 to 15.6; number 5 to 7.9; number 6 to 3.9 and number 7 to 1.9 µg/ml. This correction was included at figures and text.

d) Figure 4. The activity of 31,2 µg/m of F4 Petiveria alliacea in the figure 4 B y 4 C is not coincident.

• Several experiments support that F4 fraction tumor cell treatment causes G2 arrest. Significant differences between treated cells and negative control (ethanol) are now illustrated in Figure 5A. All cells were synchronized at G1 phase before treatment, nonetheless inherent differences in biological experimentation, could explain the difference in percentage observed in cells arrested at G2 phase as shown in the new figures 5B and 5C.

e) What is the hypothesis for justify the differences between the different concentrations evaluated. At low concentration, the effect is more important.
- Cycle arrest of cells treated with high concentrations of F4 fraction might not be spotted due to toxicity exerted over the cytoskeleton, causing cell death.

f) The legend of this figure is very long.
- Figure legend was abbreviated as suggested.

g) Proteomics. The authors must be indicated the tumoral cell line studied by this methodology.
- In proteomics section tumor cell line name was included.

h) I’m consider essential know what is the protein expression pattern in this normal cells incubated with F4 Petiveria alliacea.
- In this particular experiment the “normal” protein expression pattern considered was tumor cells treated with the vehicle. In the article it is now clearly explained. Change in protein expression was evaluated by comparing tumor cells with and without treatment. Analysis of down or up regulated proteins was carryout calculating the ratio between treated and non-treated cells. Ratio values lower than 0.75 and higher than 1.25 was considered as down or up regulated proteins respectively, as clearly explained in methods section of the article.

5) Conclusion. “In conclusion, our study demonstrates that ethyl acetate soluble extract from Petiveria alliacea, F4 fraction, exhibits multiple antitumoral activities against human and mouse tumor cells. F4 fraction exerts G2 cell cycle arrest, induced actin cytoskeleton reorganization, disturbed morphology and together with DNA fragmentation and a decrease in clonogenicity, our studies suggest that F4 fraction have different molecular targets to exert its antitumor activity being a good candidate to be tested in phase I clinical trials.”

This conclusion must be revised. Differents assay have been realized against each cellular type. Generalize not ever is possible.

...."good candidate to be tested in phase I clinical trial".

“good candidate to be tested in preclinical assays”

- The conclusion have been revised and accounted for all suggestions. The manuscript clearly specifies that the present study is not in clinical phase.

- Minor Essential Revisions

6) Background (not Introduction), Methods (not Materials and methods)
- Introduction have been replace in the text for background as well as Materials and Methods for Methods
7) Conclusión (falta)

- Conclusion has been addressed as part of the discussion.

**Reviewer: Federico Garrido**

**Major Compulsory Revisions**

1) The results of proliferation/cytotoxic assays, including curve dose/respond, with different tumour cell lines must be presented. There is no figure with these results.

- The IC50 for F4 fraction was calculated for each cell line treated and plotted into a new graph were dose-response curves for all tumor cell lines and normal cells are shown.

2) Apoptosis must be measured and quantified using the Annexin V assay.

- Currently at our lab we are standardizing all suggested protocols to evaluate Apoptosis, including Anexin V test. However, is interesting to point out that clonogenicity test is a gold standard technique to assure tumor cell death, reason why this technique was used in our study.

3) The statistical analysis of the results would be performed: the error bars must be plotted in each figure.

- All statistical analyzes were carryout and error bars plotted at the correspondent graph.

**Minor Essential Revisions**

4) The figure sizes would be changed: the figures are very small.

- Figures are small in order to fit in a letter size paper.

**Reviewer: Adolf Nahrstedt**

**Major:**

1) Authors should bed more self-critical and clearly state that their tests all are in vitro. Authors go too fast when they argue next to do are clinical studies phase I; what they need before are preclinical studies in animals. It should be clear that the used fraction F4 was by far not tested for its pharmacokinetic properties.

- The manuscript was revised and explicitly stated that all tests were carryout in vitro. Currently, we have preliminary evidence that the fraction has in vivo activity. Finishing the molecular targets study, we plan to continue with in vivo phase.
However, the text was revised and account for all suggestions. However, we never specified that the present study was in clinical phase I.

2) Authors should also correctly name their fraction as F4, but not "EtOAc extract" or "complex extract" or "partially purified" and so on. F4 should be explained early in the manuscript as a fraction obtained by RP18 chromatography of the EtOAc soluble material of a water diluted EtOH extract.

- The different names given to the F4 fraction were removed. The fraction preparation is explained under Methods.

3) Authors should also early in the manuscript explain that their original EtOH extract was obtained from leaves and stems of the title plant.

- In methods is clearly written that stems and leaves dry and ground were used for the study.

4) Concerning the phytochemical characterization: All data are pretty tentatively. This should be emphasized! The present version may give the information to less informed readers that the authors have clarified the addressed constituents.

- We agree that phytochemical characterization is tentative. Currently we are isolating each fraction compound to verify its identity. In the manuscript was clearly stated that the phytochemical data is tentative.

5) Authors do not explain why they select the F4 fraction for their investigations. This should convincingly be added.

- Biological analyzes were carryout in all plant fractions, and F4 fraction was the one exhibiting the most outstanding antitumor activity. This clarification was included in the text to explain the reason why we choose de mentioned fraction.

Minor:

6) Did the authors prove whether their MTT test showed interference with the F4 fraction compounds? (see Planta Med 2002; 68: 445-8).

- Interference between compounds in F4 plant fraction and MTT were avoid because F4 fraction is carefully remove before adding MTT as suggested by Bruggisser (Planta Med. 2002 68(5):445-8).

7) Introduction: Cystein sulfoxides are not novel! In fact they are well known from i.e. Alliacean plants

- We knew the compound was already reported for the Petiveria. The appropriate corrections were added to the text.
8) *Petiveria alliacea* is by far not “fully phytochemically characterized”. Authors may tell the reader, which percentage of a crude EtOH extract is known and which is not known.

- In the manuscript was specified that *Petiveria alliacea* has not been completely characterized. Concerning the percentage of ethanol extract that has been studied, we haven’t found any reference that states such value at the consulted plant reports.

9) **Math+Meth:** The correct elution volumes of the F4 fraction should be presented for readers who want to repeat the present results. Also, the volumes of eluents used for RP18 elution should be given.

- Volumes of the eluents were included in the text

10) **Results:** What is "senfol"?

- Senfol corresponds to 1,2 diisothiocyanato ethane.

11) It sounds strange to name allantoin an alkaloid.

- Naming allantoin as alkaloid was removed from the text.

12) Authors pretty brave give pinitol as probably present. This is one stereoisomer out of 9! Why just pinitol? Further, are they convinced that this well water soluble compound will be present in the F4 fraction of an EtOAc soluble fraction?

- Pinitol is a compound widely reported to be present in the plant. Compound identification was carryout by comparing compounds masses with literature reports and with this methodology is unfeasible to state type of isomers. This situation was explained in the text.

13) What do authors mean when they speak of a "whole extract" of P.a.?

- The initial ethanolic extract without fractionation.

14) There are several printing errors in the manuscript; e.g. citotoxicity, *Petiveria* with a capital or a small P in the sentence; it should read leridol 5-methylester; etc, etc.

- Printing errors were corrected in the text

We hope to have addressed all reviewers’ suggestions to your complete satisfaction.

Sincerely,

Susana Fiorentino. PhD