Author’s response to reviews

Title: In vitro and in vivo safety evaluation of Dipteryx alata Vogel extract

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Author’s response to reviews: see over
Dear Miss Puebla,

We would like to thank you for considering our manuscript for publication, and especially by the revision performed by referees. They have contributed to improve the text clarity and quality.

Please, consider the enclosed revised manuscript “In vitro and in vivo safety evaluation of *Dipteryx alata* Vogel extract” for publication in BMC Complementary and Alternative Medicine. All named authors have seen and agreed to the revised version of the paper and the person included in the acknowledgements section has agreed to his inclusion.

We have also enclosed the answers to the reviewer’s queries. We hope you find them appropriate. Otherwise, please let us know. We will be glad to answer any future inquiries. The changes we have made on manuscript text are in red. The revised manuscript is now in conformity to the journal style.

Sincerely,

Dr Eliana Aparecida Varanda
First Reviewer's report

Reviewer: Iwonna Rahden-Staron

Re: Manuscript The article “In vitro and in vivo safety evaluation of *Dipteryx alata* vogel extract” by Mencacci Esteves-et al. describes lack of mutagenic activity and lack of effects on the pregnancy of rats.

The reviewed manuscript is original, well presented, and a great amount of work done. Since in Brazil, medicinal plants are widely used by the people, which leads to a constant requirement for toxicity tests to be performed on the plant extracts, these findings are potentially of interest. The study is well conducted and the results are important in view of the level of human exposure, especially in Brasil, and the limited results regards the mutagenicity and toxicity of tested plant extract on mammal organism.

The objective of presented study was to evaluate the safety of *D. alata* barks extract. The authors used two approaches. Vegetal drugs of *D. alata* barks were submitted to quality control assays and further to the safety assays under 1) in vitro parameter by *Salmonella* (Ames) mutagenicity, and 2) in vivo parameter on the pregnancy of rats.

The authors show that the extract was non-mutagenic to any of the assessed strains TA97a, TA98, TA100 and TA102 even after metabolic activation (+S9). All in vivo parameters (reproductive ability evaluation, physical development of rat offsprings, and neurobehavioral development assays) showed no changes related to control group.

While the paper contains some useful information, I have the concern that need to be addressed before it is accepted for publication.

I have the following specific comment: the recommended set of bacteria strains (OECD) according to Guideline for industry. Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, April 1996, ICH S2A (see: OECD Guidelines for the testing of Chemicals, Bacterial Reverse Mutation Test 471, 1997) needs additional *Salmonella typhimurium* TA1535 strain. So, the authors should complete their study with that strain.

-The OECD recommends at least five strains (*see text below), and we have used four strains (TA97a; TA98; TA100 and TA102). Besides, according to OECD, even adding TA1535 to TA97a; TA98 and TA100, they may not detect certain oxidizing mutagens, cross-linking agents and hydrazines, which in turn may be detected by TA102. Thus, the absence of TA1535 in the present study did not invalidate the conclusion about the safety of *D. alata* extract. In addition, other studies (Sueiro RA, Garrido MJ, Araujo M. Mutagenic assessment of Prestige fuel oil spilled on the shore and submitted to field trials of bioremediation. Sci Total Environ. 2011; Ben Sghaier M, Boubaker J, Skandrani I, Bouhl el I, Limem I, Ghedira K, Chekir-Ghedira L. Antimutagenic, antigenotoxic and antioxidant activities of phenolic-enriched extracts from Teucrium ramosissimum: combination with
their phytochemical composition. Environ Toxicol Pharmacol. 2011 Jan;31(1):220-32) were also carried out using only four or three strains.

*At least five strains of bacteria should be used, including four strains of S. typhimurium (TA1535; TA1537 or TA97a or TA97; TA98; and TA100) that have been shown to be reliable and reproducibly responsive between laboratories. These four S. typhimurium strains have GC base pairs at the primary reversion site and it is known that they may not detect certain oxidizing mutagens, cross-linking agents and hydrazines. Such substances may be detected by E. coli WP2 strains or S. typhimurium TA102 which have an AT base pair at the primary reversion site (see: OECD Guidelines for the testing of Chemicals, Bacterial Reverse Mutation Test 471, 1997).

Second Reviewer’s report

Reviewer: Alvaro Augusto da Costa Leitão
The question is well defined by the authors
The methodology is appropriate and has been well described for testing the quality and toxicity of the extract, however, the description of the mutagenicity tests needs some corrections:

Second paragraph of “Biological Tests”
1 - In the composition of the top agar, histidine and biotin (0.5 mM each) must be Added

-Histidine and biotin (0.5 mM each) were included in the composition of the top agar.

2 - Define minimal agar and explain why you used this technique (double layer), which is not described in the work of Ames.

-We agree with this observation and the text was corrected (pag.6). In fact we used the technique described by Maron and Ames (1983): After 20 min of incubation at 37°C, 2 mL of molten top agar (0.6% agar, histidine and biotin 0.5 mM each, and 0.5% NaCl) was added and the mixture was poured on to a plate containing minimal glucose agar (1.5% Bacto-Difco agar and 2% glucose in Vogel-Bonner medium E).

Other general methodological issues:
1 - Why the lyophilized preparation that was used in vivo was not used for mutagenicity?

-The same lyophilized preparation used in vivo was also used for mutagenicity. This question was cleared in Materials and Methods (pag.4).

2 - Why was DMSO used as a negative control? A water-alcohol solution should have been used.
As a water-alcohol solution was not used as vehicle, since the same lyophilized D. alata was used in in vivo experiments, there is no need to include it as a negative control. Besides, DMSO is a well standardized negative control.

The results are relevant and the manuscript is clear and fairly well describes the results. However, in the “Results and Discussion” section the authors need to correct the following:

- Shortening the second paragraph,

  -We have revised and shortened the second paragraph as suggested.

Delete the third paragraph (the explanations are not necessary)

-We have deleted the third paragraph as suggested.

The discussion and conclusions are well addressed, except:

Fifth paragraph: The results only allow to state that the extracts are not mutagenic. The authors cannot state that they are not carcinogenic.

-We agree and we have changed the statement as suggested.

General comment
The toxicological tests were well conducted and the authors should have mentioned his work “Implementation of the three Rs in the human hazard assessment of Brazilian medicinal plants: An evaluation of the genotoxic potentials of cititoxic and Diptryxalata Vogel in ATLA Alternatives to Laboratory Animals, 33:189-196 2011.

-We have included a mention of the suggested study as citation [9].

Third Reviewer's report
Reviewer: Israel Felzenszwalb

The authors should review the species name following the correct one. Genus in caps and the second name (species, minus).

-The species names were corrected in the text.

Introduction section: In the last paragraph, the authors written "all these properties". Which one? There is no one mentioned in the introduction.

-The D. alata properties were mentioned in the first paragraph: “…. Its fruits are consumed by cattle and wild animals [2] and as sweetmeat by humans [3]. Its seeds are edible,
nutritive and the oil has medicinal properties \cite{1,2,4}, whereas other parts from plant are popularly used as anti-rheumatic, tonic and menstrual regulator \cite{5}.

The species D. alata Vogel (year is absent). Please include it.

-The taxonomy of D. alata is correct, since Vogel is the name of botanic author. In this case it is not necessary the year.

More information about the issue can be found in:
http://www.tropicos.org/Name/13000476

*Dipteryx alata* Vogel
Dicot Family: Fabaceae
Authors:
*Vogel, (Julius Rudolph) Theodor*

Material and Methods: It is necessary a precise time of preincubation in Ames test. Toxicity should be performed and included in the Ames test table.

-The time of preincubation was changed to 20 minutes in Material and Methods.
In both plate incorporation and pre-incubation methods, the top agar culture medium contains just enough of the essential amino acid for the bacteria to divide a few times resulting in a background lawn of minute colonies. Any bacteria that mutates, either spontaneously or as a result of treatment with the test substance will carry on dividing and form a visible colony. The spontaneous mutation rate depends primarily on the bacterial strain but is re-assessed in each experiment using a concurrent control culture which has been treated with the solvent only. As with all genotoxicity tests, concurrent solvent/vehicle and positive controls are included in each experiment. Toxic effects result in poor growth of the background lawn or a substantial reduction in the spontaneous mutation rate.

The reduction in the number of His\(^+\) revertants can be checked in the Ames test table by the comparison of the values of mutagenic index. We can observe that the smallest values of mutagenic index were around 1.0 showing that the extract not decreases the spontaneous levels of revertants and the background lawn is normally checked in all the experiments.