Author’s response to reviews

Title: Vellozia flavicans Mart. ex Schult. hydroalcoholic extract inhibits the neuromuscular blockade-induced by Bothrops jararacussu venom

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Version: 2 Date: 30 July 2013

Author’s response to reviews: see over
July 30, 2013

Dear Prof Hideaki Hara
Associate Editor from Biomed Central Editorial
ditorial@biomedcentral.com

MS: 7131557959876314 – “Medicinal potential of Vellozia flavicans Mart. ex Schult.: an inhibitor of the neuromuscular blockade-induced by Bothrops jararacussu venom”

We are grateful all reviewers’ comments on our paper now entitled “Vellozia flavicans Mart. ex Schult. hydroalcoholic extract inhibits the neuromuscular blockade-induced by Bothrops jararacussu venom”. We made the suggested corrections to meliorate it and also, when necessary, the modified text was added in the manuscript.

Bellow our answers to Reviewers' comments.

Reviewer's report
Title: Medicinal potential of Vellozia flavicans Mart. ex Schult.: an inhibitor of the neuromuscular blockade-induced by Bothrops jararacussu venom
Version: 1 Date: 10 June 2013
Reviewer: Stella R Zamuner
Reviewer's report:
Comments to the Author
Major Compulsory Revisions: The paper is interesting but numerous points should be addressed before its consideration for publication.

General:
- The English is generally poor. The manuscript design is confusing. There is a general discontinuity in the text.

Answer to Comment 1: The language was improved, as suggested, by a native speaker. Abstract, Introduction and Discussion was rewritten. The manuscript design was improved aiming to clear the confusing points.

- This study intends to provide information on the effect of Vellozia flavicans plant extract in neuromuscular blockade caused by B. jararacussu venom and antimicrobial activity of this plant. It is not clear the connection between neuromuscular and microbicidal activities of the plant, since the neuromuscular effect is systemic and microbicidal activity is a local effect. It should be explained.

Answer to Comment 2: We agree with this question. The 2nd paragraph of Introduction already comprises the antimicrobial study justification (of Vellozia flavicans extract). See: “… and skin infection with abscess, usually associated with inoculation of bacteria present in the mouth of the snake, favoring the development of necrosis, which can develop into gangrene and amputation [2].”

Thus, we inserted a new paragraph in Introduction (3rd paragraph) about the findings on neurotoxicity signs in Bothrops envenomation justifying the use of in vitro neurotoxic study.
“There are few data in the literature on the in vivo neurotoxicity induced by B. jararacussu venom, some of them as unspecified signs [3, 4, 5] or related to the systemic effect including blindness, blurred vision, difficulty in swallowing and paralysis, reminiscent of the action of Crotalus venom [6]. However, the well known in vitro irreversible neuromuscular blockade induced by B. jararacussu venom firstly showed by Rodrigues-Simioni et al. [7] has inspired more studies with other species of Bothrops genus.”

- The authors intend to investigate whether the V. flavicans plant has anti-snake properties (or antophidian potential), however only one effect caused by the venom was investigated, the neuromuscular blockade, effect which is not characteristic of bothropic snake venom, but characteristic of crotalic snake venom. The anti-snake properties should be corrected by anti-neuromuscular effect or clarified further by the authors.

**Answer to Comment 3:** The new inserted 3rd paragraph (above) intends to correct it. Also, in order to clarify the objectives of this study the 6th paragraph of Introduction was rewritten to:

“Plants used popularly as anti-inflammatory can be potentially effective to treat snakebites. This pharmacological relation has been confirmed by studies which found that some plants extracts and their compounds can exhibit both anti-inflammatory and anti-snake venom properties [18, 19]. Therefore, as V. flavicans has been used popularly as anti-inflammatory, it would be interesting to investigate whether this plant possesses anti-snake venom activity. For answering this hypothesis Bothrops jararacussu venom was reasonably chosen since it causes inflammation at the bite site, differently of Crotalus genus, with the advantage of inducing an irreversible in vitro, but not in vivo, neuromuscular blockade [7]; a parameter experimentally recognized and scientifically validated.”

In Abstract, the Background was rewritten to:

“Snakebite is a prominent public health problem in tropical countries. The genus Bothrops is one of the most important in causing snake envenomation triggering local and systemic effects in its victims such as edema, pain, erythema, cyanosis, infections and necrosis. Vellozia flavicans plant has been used popularly in the Brazilian cerrado as an anti-inflammatory and inflammation is one of clinical signs rapidly developed by Bothrops genus at the bite site; hence this plant may potentially possess anti-snake venom and/or antimicrobial activities, objectives of this study.”

**Specific comments:**

In the abstract session:

- The authors mention the profile of the chromatography of plant extract. What is the reason to do the chromatography if in the entirely study the authors used the total extract and the concentration of each component of the plant is not known? This point should be clarified.

**Answer to Comment 4:** In several points of manuscript the reason to do the Thin Layer Chromatography were justified. See bellow:

**Abstract (Methods):**

“The chromatographic profile of V. flavicans extract was obtained via Thin Layer Chromatography (TLC) in order to detect the main constituents according to the literature;…”

**Introduction:** (last paragraph was rewritten)

“Considering its potential as anti-snake venom, the present study aimed to evaluate the activity of V. flavicans extract to reverse the in vitro neuromuscular blockade caused by B.
Jararacussu venom in mice. In addition, antimicrobial activity of this plant was tested to verify if its extract could also be used against bacterial infection caused by snake bite and other types of infections, expanding the medicinal potentialities of this plant. Qualitative scrutiny of V. flavicans extract was also carried out via Thin Layer Chromatography (TLC) for qualitative analysis of the main classes of constituents.”

Results and discussion (a new paragraph was inserted to connect the use of TLC)
(1st and 2nd paragraphs in revised manuscript)

“One of the main criteria to select plants for pharmacological studies is their traditional use in folk medicine (ethnopharmacology). Plant selection is also based on chemical composition mainly for certain compounds from a defined chemical class with a known pharmacological activity [27, 28]. In this study both criteria were adopted: ethnopharmacology (since V. flavicans have anti-inflammatory and antirheumatic properties) and chemical composition [flavonoids (phenolic compounds), diterpenes and triterpenoids (“terpenoid”, a term which is used to indicate that all such substances have a common biosynthetic origin, isoprene molecule) were the main secondary metabolites (phytochemicals groups) isolated from Velloziaceae family]. Flavonols are related to natural resistance factors and possess biological effects including antimicrobial and cardiovascular activities [29], whereas terpenoids are related to different functions including growth-regulating properties, communication and defense among insects [21].”

(3rd paragraph in revised manuscript)

“After steps as collection, botanical identification, stabilization, powdered and extraction process, the plant extract was first qualitatively analyzed by TLC [30], in order to obtain a chromatographic fingerprint, since TLC plate provides suggestive evidence of plant chemical composition.”

(5th paragraph in revised manuscript)

“Even using other solvent systems, terpenoids were not visualized when compared to its representative ß-sitosterol, the most abundant of the phytosteroids. Therefore, this chromatographic profile characterizes V. flavicans extract used in this study and works as a plant quality control, since allows comparisons with data from the literature. On the other hand, from the botanical point of view the pharmacological activity can be attributed mainly to flavonols.”

-line 4 “Vellozia flavicans has been….”, should be replaced by “Vellozia flavicans plant has been….”.

Answer to Comment 5: We thanks for correcting it. The word plant was added in the text.

In the Introduction session:
- would be interesting to describe a little more the neuromuscular blockade caused by bothrops snake venom and the importance of it for the bothropic accident.

Answer to Comment 6: As above mentioned a new paragraph was included focusing the neurotoxicity of Bothrops jararacussu (3rd paragraph).
There are few data in literature on the in vivo neurotoxicity induced by B. jararacussu venom, some of them as unspecified signs [3, 4, 5] or related to the systemic effect including blindness, blurred vision, difficulty in swallowing and paralysis, reminiscent of the action of Crotalus venom [6]. However, the in vitro irreversible neuromuscular blockade induced by this venom firstly showed by Rodrigues-Simioni et al. [7] has inspired subsequent studies with other species of Bothrops genus.”

In the Results and discussion session:

Page 7 – 3 paragraph: “These results suggest the beneficial effects of higher concentration of V. flavicans at skeletal apparatus, but the basal response decrease at lower concentration may suggest a parallel regulation on skeletal excitability since other endogenous signaling pathways are capable of compensating the cholinergic control of movement, as glutamate.”

This paragraph is not clear.

Answer to Comment 7: More information was added to the paragraph.

“These results suggest the beneficial effects of higher concentrations of V. flavicans at skeletal apparatus, but the basal response decrease at lower concentration may suggest a parallel regulation on skeletal excitability, by an unclear mechanism, since other endogenous signaling pathways are capable of compensating the cholinergic control of movement, as glutamate, recently suggested as being a co-transmitter of acetylcholine in motoneurons from mammalian neuromuscular junction [38, 39].”

Answer to Comment 8: The peripheral-type glutamate receptor was found at mammalian JNM; this receptor used to be accepted as exclusively participating in NMJ from invertebrates, where glutamate acts as a major excitatory neurotransmitter (Lunt and Olsen, 1988). However, recent research has proposed the participation of glutamate in modulating cholinergic transmission (Brunelli et al., 2005), suggesting glutamate as a possible co-transmitter of ACh in motoneurons (Waerhaug and Ottersen, 1993; Meister et al., 1993). The application of glutamate to strips of rat diaphragm has been shown to contribute to the maintenance of the resting membrane potential (Urazaev et al., 1999) and to inhibit the nonquantal release of ACh from nerve endings (Malomouzh et al., 2003). Since the expression of glutamate receptors at skeletal muscle endplates gave contradictory results (Kraus et al., 2004; Boulland et al., 2004), our results may have been influenced by a glutamate response in JNM, by a still unclear mechanism, and these findings open questions arising from transmission and co-transmission concepts.


Level of interest: An article of limited interest
Quality of written English: Needs some language corrections before being published
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's report

Title: Medicinal potential of *Vellozia flavicans* Mart. ex Schult.: an inhibitor of the neuromuscular blockade-induced by *Bothrops jararacussu* venom

Version: 1  Date: 4 July 2013
Reviewer: Maria Alice Cruz-Hofling

**Reviewer's report:**

Major compulsory revisions:

Firstly, looking at the literature one sees that *Bothrops jararacussu* has a direct myotoxic action which induces a very fast onset of myonecrosis. The effects at the snakebite site involve muscle fiber membrane disruption and subsequent profound fiber alteration in conjunction with local microcirculation failure. Along with this, there is migration of neutrophils and macrophages to the site of venom presence and inflammation. Despite the peripheral nerve fibers inside the muscle show morphological abnormality (seen experimentally), clinically, there is no report on neuromuscular paralysis in victims of *B. jararacussu* accident (Milani et al., 1997). In line with literature, in the second sentence of the Abstract and the second sentence of the second paragraph (page 3) of the Introduction, the authors did not include neurotoxicity or neuromuscular paralysis among the envenomation manifestations exhibited by human victims of accidents. A clear hypothesis and objective of the work is needed.

**Answer to Comment 1:** The 2nd paragraph of Abstract was rewritten clarifying the *in vitro* nature of neuromuscular blockade-induced by *Bothrops jararacussu*, as following:

> “The chromatographic profile of *V. flavicans* extract was obtained via Thin Layer Chromatography (TLC) in order to detect the main constituents according to the literature; the anti-snake potential was measured by its ability in neutralizing the known *in vitro* neuromuscular blockade caused by *Bothrops jararacussu* venom using mouse phrenic nerve-diaphragm model; and the antimicrobial activity was assayed against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* strains, by means of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods according to Clinical and Laboratory Standards Institute (CLSI) guidelines.”

In Introduction, a new paragraph (3rd) about neurotoxicity of *Bothrops jararacussu* venom was inserted in the text:

2nd paragraph

> “In Brazil, the *Bothrops* genus sp. is part of major groups of snakes causing envenoming. The accident caused by snakes of this genus, *including Bothrops jararacussu*, is characterized by local and systemic effects such as inflammatory activity at the site of the bite, ecchymosis, bleeding and skin infection with abscess, usually associated with inoculation of bacteria present in the mouth of the snake, favoring the development of necrosis, which can develop into gangrene and amputation [2].

3rd paragraph

> “There are few data in the literature on the *in vivo* neurotoxicity induced by *B. jararacussu* venom, some of them as unspecified signs [3, 4, 5] or related to the systemic effect including blindness, blurred vision, difficulty in swallowing and paralysis, reminiscent of the action of *Crotalus* venom [6]. However, the well known *in vitro* irreversible
neuromuscular blockade induced by this venom firstly showed by Rodrigues-Simioni et al. [7] has inspired more studies with other species of Bothrops genus.”

Secondly, based on this, the title of the manuscript also seems inappropriate since it implies a potential medicinal use of Vellozia flavicans extract against neuromuscular blockade induced by B. jararacussu venom, which apparently does not occur in human victims. Authors need to choose another more adequate title.”

Answer to Comment 2: The title was change to:

**Vellozia flavicans** Mart. ex Schult. hydroalcoholic extract inhibits the neuromuscular blockade induced by *Bothrops jararacussu* venom

An important point, is that the *V. flavicans* leaves extract needs to be pre-incubated with *B. jararacussu* venom to abolish the neuromuscular blockade. It is important to incubate the preparation with *B. jararacussu* venom until partial neuromuscular blockade then wash the preparation and then incubate it with *V. flavicans* extract? If the partial blockade induced by venom was reverted the protection promoted by the extract will be confirmed.

Answer to Comment 3: This suggested protocol is very interesting, but our experience with this venom has shown that the cell damage level induced by *B. jararacussu* venom compromises irreversibly the muscular response measured by myographical parameters at the same level. Except in the pre-incubation model, the extract addition after some blockade level induced by *B. jararacussu* venom only inhibits the damage progress, as shown here in a model simulating the accident sequence, i.e., snakebite followed by therapy beginning.

Also, it is important to clarify why to choose the venom of *B. jararacussu* which is myotoxic instead of the venom of a species with neurotoxic venom.

Answer to Comment 4: *Bothrops* venom has inflammatory action at the bite site, differently from the neurotoxic venom as from *Crotalus* genus. It was added in the text at the 6th paragraph of Introduction:

“Plants used popularly as anti-inflammatory can be potentially effective to treat snakebites. This pharmacological relation has been confirmed by studies which found that some plants extracts and their compounds can exhibit both anti-inflammatory and anti-snake venom properties [18, 19]. Therefore, as *V. flavicans* has been used popularly as anti-inflammatory, it would be interesting to investigate whether this plant possesses anti-snake venom activity. For answering this hypothesis *Bothrops jararacussu* venom was reasonably chosen since it causes inflammation at the bite site, differently of *Crotalus* genus, with the advantage of inducing an irreversible *in vitro*, but not *in vivo*, neuromuscular blockade [7]; a parameter experimentally recognized and scientifically validated.”

In the section “Conclusion”, we also clarified the type of neurotoxicity (*in vitro*) exerted by venom.

“*In conclusion, the present study found a promisor antiophidian ability to *V. flavicans*, as we demonstrated that its extract was able to protect nerve-muscle preparations against *in vitro* neuromuscular blockade induced by *B. jararacussu* venom.”

In this sense, I suggest that the authors emphasize the contributions of this study by showing any possible practical application of the knowledge provided by the current findings.
Answer to Comment 5: This suggestion was included at the last paragraph before conclusion:

“However, although *V. flavicans* extract did not show any antimicrobial activity these results does not reduce its potential as a promisor extract to treat snake envenomation, since possible practical application of the knowledge by the current findings would be, in theory, minimizing the local inflammation and/or avoiding the cell damage progress.”

In the minor comments, I have pointed out some mistakes in the text.

Minor essential revisions:
1. Since possible correspondences will be sent to Brazil, I believe that the name of the institutions, affiliations and Zip Code should be written in Portuguese.

Answer to Comment 1: Thank you. All suggestions were adopted in the manuscript.

2. In the Results of the Abstract is written “…the irreversible neuromuscular blockade induced by *B. jararacussu.*” The snake causes neuromuscular blockade?

Answer to Comment 2: The word “venom” was added after *B. jararacussu*.

3. Also in the Results of the Abstract is written “…in a model simulating the accident the extract was totally….”. This is an in vitro model of phrenic nerve diaphragm preparation and this reviewer does not see how this model simulates an accident with *B. jararacussu*.

Answer to Comment 3: In the Results of the Abstract the text was change to:

“In a model with no previous incubation where the venom was added into the bath during 10 minutes followed by extract addition, there was total inhibition of the neuromuscular blockade progress.”

In Results and Discussion, this question was also rewritten:

“Figure 4 shows the representative myographical register of *B. jararacussu* venom (A, 40 µg/mL) that exhibits the characteristic irreversible paralysis. When venom (40 µg/mL) + *V. flavicans* extract (1 mg/mL) are preincubated for 30 min before addition into the bath, the toxic effect of the venom no more was expressed (B), showing the total protection exerted by plant, by a mechanism that excludes protein precipitation as seen to tannins and tannic acid [43]. Even in preparations pretreated with *B. jararacussu* (40 µg/mL) during 10 min (when contracture and an installed 40% of paralysis were visible), the addition of *V. flavicans* extract into the bath (1 mg/mL), in a model with no preincubation, the plant was able in hindering the paralysis progress (C). The mechanisms by which *Bothrops jararacussu* induce *in vitro*, but not *in vivo*, neuromuscular paralysis were recently compiled [34] and, experimentally, there is a direct relation between the cell damage level and the percentage of twitch tension response.”

4. In the Conclusion of the Abstract is written “ *V. flavicans* extract possess anti-snakebite potential…” Does the extract prevent the snakebite? Or would be against the effects of snakebite? (the correct use of the verb is “*V. flavicans* possesses…””)
Answer to Comment 4: The text was change to:

“These present results indicate that the *V. flavicans* extract possesses antiophidian potential, but not antimicrobial, as it protected nerve-muscle preparations against *in vitro* neuromuscular blockade induced by *B. jararacussu* venom.”

5. There are non-venomous snakes, and then I suggest adding the adjective. For example, “Envenoming subsequent from venomous snake attacks....”

Answer to Comment 5: Thank you!

6. Unconventional use of the English should be avoided. For example, in the Author’s Contribution, the word execution is unconventional (“execution” is more used to indicate the act of killing someone especially as a legal punishment; also the word “redaction” should be replaced by “writing”).

Answer to Comment 6: All right!

In conclusion, the authors should re-direct their objective, such as simply investigate if the extract of *V. flavicans* leaves abolish the neuromuscular blockade produced in vitro in a murine nerve-muscle preparation. Eventually, they could end the manuscript with a conclusion on the possible application of their findings.

Answer to Comment 7: It was made! Thank you!

I believe that the authors could make a fully revision to the paper and, then, resubmit it for new appreciation.

Answer to Comment above: All suggestions were appropriate and rigorously considered.

Discretionary revision

Since *V. flavicans* leaves allegedly have anti-inflammatory effect why not to test the extract of *V. flavicans* leaves against the inflammatory response induced by the venom?

Answer to Comment above: We argue this question on 6th paragraph of Results and discussion in the revised manuscript, as following:

> “Mors et al. [32] declare to have a striking parallelism between the ability of plants and their chemical components of neutralizing the actions of snake venoms, and anti-inflammatory and anti-hepatotoxic properties. Here, we selected the *B. jararacussu* venom for testing the first premise, i.e. that an anti-inflammatory plant has an antiophidian potential, since this venom causes inflammation at the bite site and also induces an *in vitro* irreversible neuromuscular blockade effect as already well established since 80’s [7, 33, 34]. The clinical effects of this venom were very well described by Milani Júnior et al. [35] and are similar to other *Bothrops* genus, with severe signs of local and systemic envenoming. Although the mechanism by which *Bothrops* venoms induce cytokine production is not totally understood [36], the agents that lead to inflammation (a signal- mediated response to cellular insult by infectious agents, toxins, and physical stresses) are well known. Indeed, as the anti-inflammatory action was already attributed to *V. flavicans* [8], we addressed a rationale experimental design focusing on neuromuscular junction area and also to antimicrobial...
potential of *V. flavicans* extract. To support this study, World Health Organization has included snake bite envenoming as a neglected disease [37], and few efforts have been made to change this scenario in the world, even with the serum therapy existence. Thus, the plant kingdom can be exploited for complementing the serum therapy.”

**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:** No, the manuscript does not need to be seen by a statistician.  
**Declaration of competing interests:**  
I declare that I have no competing interest.

Looking forward to have the manuscript accepted for publication, I thank you for your time.

Sincerely yours,

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