Author's response to reviews

Title: TRPV1 channel inhibition contributes to the antinociceptive effects of Croton macrostachyus extract in mice

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Author's response to reviews: see over
From: NGUELEFACK Télesphore Benoît
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To: The Editor-in-Chief
    BMC Complementary and Alternative Medicine

Submission of a manuscript

Dear Sir,

On behalf of all other co-authors, I am very pleased to resubmit the manuscript entitled “TRPV1 channel inhibition contributes to the antinociceptive effects of Croton macrostachyus extract in mice” that has been amended as requested by the reviewer.

We would like to thank the reviewers that critically evaluated the present paper.

Reviewer 1
1) Von Frey measurements starting 1h after CFA injection seems a bit awkward in terms of kinetics for CFA to induce inflammatory process. Measurements are often done 24 – 72h after CFA injection. Proper background and references are needed to justify why measurements started as soon as 1h after CFA delivery.

Answer: It has been shown by Sotocinal et al. (2011) that pain behavior appears as early as one hour post CFA injection (Please see Sotocinal et al. Molecular Pain 2011, 7:55). Our protocol was drawn based on this result.

2) On figure 1 and on page10, lines 206-207 authors mentioned that treatment induced an progressive and long lasting effect, however it took 2 days to watch effect again after the daily treatment stated (no effect on 7 and 8 days), indicating development of a tolerance phenomena what can be seem on the graphic. Mention about this phenomena must be added to the section, with does not mean that it will compromise the publication of the manuscript, in fact the lack of such info might be an issue. This should be also mentioned in the discussion section.
**Answer:** We do not think there is a tolerance phenomenon here. We talk about tolerance when repeated administration of a substance results in a reduction of its activity. In this experiment, plant extract was administered on the first day and the effect was maintained up till day 5, post administration. It is important to consider that in the present experiment, the effect of each administration was evaluated 24 hours later. As in CFA model the pain becomes more resistant with the time, we believe the plant extract needs a certain level of accumulation to be able to induce a significant effect. However, it can be noticed that on days 7 and 8, there is an important antihyperalgic effect with the highest dose, although not significant. It is known that the higher the dose, the higher the tolerance. If there was a tolerance, the highest dose would have been the least potent after restarting the treatment.

3) Were statistical differences between baselines and control groups detected in every experiment? This need to be clarified and stated in all result sections of the manuscript.

**Answer:** Yes in all experiments excepted the Capsaicin test, a significant difference was observed between baselines. This fact is now appropriately introduced in result section.

Minor comments:

1) Pg2, line 29-30: rewrite “… (CFA)-induced persistent thermal and mechanical…”

**Answer:** Done

2) Pg5, lines 94-98: it is confusing the two sentences stating different animal protocols numbers, this paragraph needs to be reworded to make better sense.

**Answer:** This was a mistake. It has been corrected

3) It not clear to me why the authors have used gabapentin as a positive control for an inflammatory pain model (CFA), gabapentin is known to be used to treat neuropathic pain states in patients and not inflammatory conditions, a NSAID seems more appropriate for control in this model. Proper justification is needed.

**Answer:** It is certain that gabapentin is mainly used for neuropathic pain but it has been shown that the same is very active in various inflammatory conditions, including the CFA model (please see Gu and Huang / Pain 93 2001, 85-92; Yang et al./Molecular Brain 2012,5-18).

4) Pg6, lines 117-119. Shouldn’t the aim of the daily treatment with MECM on CFA test be done to watch development of tolerance caused by MECM treatment?

**Answer:** It was realized that unlike in CFA treated animals, the effect of the extract lasted 6 to 8 hour after administration and that no effect was even observed 24 hours after the second administration. We suspected a development of tolerance. Knowing that the maximal extract activity was observed 2 hours after extract administration, we then evaluated the effect of the extract each day at the second hour after administration. As the effect was constant all along, we could then roll out the tolerance phenomena. We can conclude that as this model of pain is robust and resistant, it needs some level of accumulation of plant extract for long lasting effect.

5) Pg7, lines 146-147: Glibenclamide is a inhibitor of ATP-sensitive potassium channels and not an antagonist, proper terminology must be used all along the manuscript.

**Answer:** Corrected

6) Pg7, line 151: intensity of 15 what? The proper unit is missing, is it 15% of total power?

**Answer:** Corrected
7) Pg7, line 155: info missing, measurements were taken up to 14 days after CFA injection, this info need to be add to the section.

**Answer**: No, in this test, animals were observed only for 24 hours. No chronic experiment was carried out.

8) Pg8, lines 168-169: again proper terminology must be used, rimonabant is not a CB1 antagonist, due to its electrophysiological properties rimonabant is an Inverse Agonist of CB1 receptors, despite its interaction with CB1 receptors shows a functionality antagonistic-like action specially when evaluated in vivo, this drug is not an antagonist, but an Inverse agonist. Proper terminology must be used.

**Answer**: corrected

9) Pg9, line 195: rewrite “… post hoc test or two-way ANOVA followed …”

**Answer**: Done

10) Pg10, line 218: change “… both extract and gabapentin…” by “… either the extract or gabapentin…”, once this seems is not a co-administration protocol.

**Answer**: Done

11) Pg10, line 227: Author state that glibenclamide failed to revers effect of MECM, but the result show it does at 4 hours. Clarification is needed.

**Answer**: The reviewer is right but since we observed a significant effect at 6 hours. We thought the effect observed at 4 hours was not relevant.

12) Pg11, line 240: Effect lasted for up to 4h, and not up to 6h as authors states.

**Answer**: Corrected

13) Pg12, line 252: rewrite “… in models of persistent inflammatory and neuropathic pain…”

**Answer**: Corrected

14) Pg13, line 282: Well stablished chronic pain after only 5 days from nerve injury? Not likely once chronic pain is observed in sciatic nerve injury models after 10 days from surgery. Reword is needed.

**Answer**: Yes, the chronic pain is well established 5 days after nerve injury. Please see Quintao et al. / Phytomedicine 2008, 15:245–252; Malmberg and Basbaum/Pain 1998, 76:215-222.

Reviewer 2
Title page
1. Standardize the membership in English the letter "d".

**Answer**: Done

Abstract
1. When referring to pain behavior in models of CFA-inflammatory and neuropathic pain induced PSNL refer to antihyperalgesia rather than antinociceptive.

**Answer**: Done
2. In the methods section (Abstract) MUST include animal species used in the study and explain better how the evaluations of mechanical hyperalgesia were performed.

**Answer:** Done

**Introduction**
1. Authors should include paragraphs to explain the reason for the choice of the mechanisms used in this study (cannabinoid and potassium channels), what their roles in pain?

**Answer:** The following paragraph has been introduced in the introduction:

Indeed, it has been shown that in both neuropathic and inflammatory pain conditions various neuronal cells proteins are up regulated, including TRPV1 receptors (3). Apart from TRPV1 antagonists, the activity of TRPV1 channels can be modulated by cannabinoid receptors which are collocated in the same nerve endings or by ATP sensitive potassium channels. These cell structures are potential targets for pain management.

**Methods**
1. To remove the sentences of lines 83-85. It should be in Sections Results.

**Answer:** We do not think so. These are data from the literature and not from the present work. Therefore, they cannot be presented in Results section.

2. On lines 95-96 to remove “(Comissao de Etica no Uso de Animais/Universidade Federal de Santa Catarina)” just let the protocol number.

**Answer:** Done

3. The reference used to justify the use of the filament of 0.4 g (Dutra et al., 2012) is inappropriate because the authors used the filament of 0.6 g, correct this information.

**Answer:** You are right, thank you for this remark. The reference Dutra et al., 2012, was replaced with Nguelefack et al., 2010.

4. To use the term intraplantar injection replacing the terms “sub plantar injection” or “under the subplantar aponevreous”.

**Answer:** Done

5. As the authors not carried out a dose response curve from which the authors pick up the doses of treatment with the plant extract?

**Answer:** Previous study from our research team guided the choice of doses (Kamanyi et al, Journal of Complementary and Integrative Medicine 2009, 6: DOI: [10.2202/1553-3840.1255](https://doi.org/10.2202/1553-3840.1255)).

6. To describe the concentration CFA was administered even 100%

**Answer:** Done

7. To correct figure legends rather than “figure legendes”

**Answer:** Done

8. To improve the figure legends

**Answer:** Done

9. To change the symbols of the subtitles leave them more readable to facilitate comprehension of the reader.

**Answer:** We do not understand what the reviewer means
Results
1. The sentence of lines 241-243, should be in the introduction.

**Answer:** This sentence introduces the hypothesis that motivated the set of experiment. We think, unless explanation, that the sentence guide the reader to better understand the work. However, similar information is now added in the introduction.

We hope the manuscript is now suitable for publication in BMC Complementary and Alternative Medicine.

Best regards

Prof. Télesphore Benoît NGUELEFACK