Author’s response to reviews

**Title:** Anti-inflammatory effect of Vaccinium oldhamii stems through inhibition of NF-κB and MAPK/ATF2 signaling activation in LPS-stimulated RAW264.7 cells

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**Author’s response to reviews:**

Response for Reviewer #1 (Yogesh A. Kulkarni)

1. Authors may have used 'stem' extract in the study, they have used the term 'branch'. The term 'branch' should be replaced with 'stem'.

RESPONSE : We replaced ‘branch’ with ‘stem’ accordingly and the revised point was highlighted as RED FONT.

2. Abstract of the manuscript is not mentioning use of leaf, fruit and extracts for the study. Abstract must be properly revised.

RESPONSE : We mentioned the use of leaf or fruit extracts accordingly and the revised point was highlighted as RED FONT.
3. What was the basis for screening extracts in inflammation? Proper justification is required to be added for selecting the plant extracts for anti-inflammatory study.

RESPONSE: It was added in Background section accordingly and the revised points were highlighted as RED FONT.

4. In methods section, the heading 'Sample preparation' should be replaced with "Preparation of extracts"

RESPONSE: We revised ‘Sample preparation’ to ‘Preparation of extracts’ accordingly and the revised point was highlighted as RED FONT.

5. Page 5, line 48, the wording "ethanol-soluble fraction" should be replaced with "ethanol extract".

RESPONSE: We revised ‘ethanol-soluble fraction’ to ‘ethanol extracts’ accordingly and the revised point was highlighted as RED FONT.

6. In methods section, authors must cite references for DPPH activity, total phenolics estimation, Griess assay and MTT assay etc.

RESPONSE: The cite references for the experiment methods were added in Method section accordingly. The revised point was highlighted as RED FONT.

7. Page 10, heading "Analysis of bioactive components" should be replaced with "Analysis of extracts". This section should be placed after section of "Preparation of extracts"

RESPONSE: We revised ‘Analysis of bioactive components’ to ‘Analysis of extracts’ accordingly and the revised point was highlighted as RED FONT.

8. How authors have identified compounds in extracts in HPLC? Whether any marker compounds were used?

RESPONSE: Anti-inflammatory compounds from VOS using HPLC were identified by the chromatogram of the analytical standards such as (+)-catechin, (-)-epicatechin, proanthocyanidin A2 and cinnamtannin. This sentence was added in Methods section.

9. Why only branch/stem extract was analysed by GCMS and HPLC?

RESPONSE: In a comparative study of anti-inflammatory activity of VOS, VOL and VOF, we found that VOS had the highest activity and selected VOS for further mechanism studies. In addition, because, the purpose of this study was to evaluate the anti-inflammatory activity of VOS and to elucidate its mechanism of action, HPLC and GC/MS analyzes were performed to identify the anti-inflammatory compounds of VOS.
10. Why leaf and fruit extracts were not included in all investigations?

RESPONSE: In a comparative study of anti-inflammatory activity of VOS, VOL and VOF, we found that VOS had the highest activity and selected VOS for further mechanism studies. In addition, because, the purpose of this study was to evaluate the anti-inflammatory activity of VOS and to elucidate its mechanism of action. We did not included leaf and fruit extracts in all investigations.

Response for Reviewer #2 (Henrique Balassini Abdalla)

1. Page 6 – Materials section (line 21-30) : This sentence should be in SDS-PAGE section (Page 8). The description of the antibodies used, as well as the primary antibody concentration and the incubation time.

RESPONSE: The description of the antibodies used in this study was added in Materials section of Methods and the concentration and the incubation time were added in SDS-PAGE and Western blot section of Methods. The revised points were highlighted as RED FONT.

2. Page 7 – Determination of NO… (line 19 – 21) : Why 6 hours of pretreatment? Its seems to be so much time. If there is any reference or data that support that this pretreatment time is necessary, it should be included in the manuscript.

RESPONSE: We pretreated the sample for 6 h so that the sample was sufficiently absorbed to the cells. In NO assay, the pretreatment time of the sample varies. For example, dopamine (1) was pretreated for 1 to 24 h and Oregano essential oil (2) was pretreated for 12 h. In addition, the treatment concentration used in this study was not cytotoxic, so the treatment time is not long.


The phrase “respectively, according to the product procedures” should be fix it. For example, “were performed accordingly with the manufacturer's protocol.”

RESPONSE: It was revised accordingly and the revised point was highlighted as RED FONT.

3. Page 8 – SDS-PAGE…. (line 40 – 42) : Please, insert the amount of protein was used per well for electrophoresis analysis (30, 50, 80 ug).

RESPONSE: We loaded 30 μg protein per well for SDS-PAGE and it was added in Method section. The revised point was highlighted as RED FONT.
4. Page 9 – PCR section: It is just a suggestion. Make a table for all your primers, it will make this section cleaner to read.

RESPONSE: We made Table 1 for the primer sequences used in this study accordingly.

5. Page 15 – Discussion (line 21 – 28): “Currently, non-steroid anti-inflammatory drugs (NSAIDs) have been prescribed for the treatment of inflammatory diseases, but the long-term use of NSAIDs is known to cause serious side effects [30]. Thus, the importance of searching for anti-inflammatory candidates with low side effects has been emphasized”. Indeed. Despite NSAIDs demonstrated potent anti-inflammatory activities, several sides effects also come together. In view of that, it will be great described the benefits of the treatment proposed here compared with NSAIDs.

RESPONSE: We tested the anti-inflammatory activity of VOS compared to TA. As a result, activity was slightly lower than TA, but considering that VOS is crude extract, VOS could be used as a material for the development of potential anti-inflammatory agents.

6. Page 16 – Discussion (line 50): Just remove the F

RESPONSE: It was revised accordingly.

7. Natural products represent an emergent field in drug development since, in theory, they possess fewer side effects rather than the available drugs. Could the authors provide some data or reference demonstrating that Vaccinium oldhamii does not have side effects related to their use?. Another point, why the authors did not perform any in vivo assay? Its is important to demonstrate this strong anti-inflammatory activity since some pharmacological agents could lose their efficacy.

RESPONSE: In cytotoxicity evaluation, it was confirmed that there is no cytotoxicity against RAW264.7 cells in the activity concentrations of VOS. In addition, some of the functional studies of Vaccinium oldhamii did not mention any side effects. Furthermore, it has been used to treat inflammatory diseases for a long time. In view of that, it is expected that the Vaccinium oldhamii has no side effects. Since we have investigated the anti-inflammatory activity of VOS and its mechanism of action in vitro studies, we will evaluate the in vivo anti-inflammatory activity of VOS in future studies.