Author’s response to reviews

Title: Psychopharmacological effects of riparin III from Aniba riparia (Nees) Mez. (Lauraceae) supported by metabolic approach and multivariate data analysis

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Dear Editor, here follows the answers to the reviewers:

Reviewer reports:
Reviewer 1: In this manuscript, the authors investigated the psychopharmacological effects of riparin III from Aniba riparia (Nees) Mez. (Lauraceae) using NMR-based metabolic approach followed by multivariate analysis.

My main comment is regarding the Results section. Results section is poorly written, difficult to follow, confusing and very repetitive. Many sentences can be shortened and improved (i.e. no need to keep mentioning ANOVA one-way analysis, applying Bonferroni post-test before presenting the data for each metabolites).

The introduction, results and discussion as well as conclusion section were modified, some sentences were indeed repetitive. Thanks for the suggestions. The modifications are highlighted in green.

Other comments are listed below;
1. Page 5 of 34, lines 123-125, the period of the day is not clear, what do you mean by 10:30h? same for line 128-129.
The tests were conducted twice a day. The time of the experiments were modified for 10:30 a.m., -3:00 GMT and 4:30 p.m., -3:00 GMT for a better understanding.

2. The quality of Figure 5 is poor and needs to be improved.

Correction has been made

3. It is much better to use the terminology scores plot and loadings plot instead of scores graphic and loading graphics throughout the text.

Correction has been made

4. No mention to Fig 5 b and c in the text

Discussion regarding Figures 5 b and C were added to the manuscript (Lines 320-323)

“The statistical analysis on Figure 5b has also demonstrated that during food restriction, when analyzing only AP and TP, the discrimination between groups was more evident, with a higher variance of AP along the PC1 dimension, and a $R^2 = 0.76$. On the other hand, during ad libitum feeding the $R^2$ correlation has diminished to 0.62 (Figure 5c), indicating that feeding reduces stress. “

5. In the discussion section, please correlate and compare your results with other works that have been done on riparin III (especially with the high dose of riparin III as the rationale of the study was to investigate the effect of low dose) or other anxiolytic

The correlation has been added to the discussion section (lines 427-433)

“Previous research have demonstrated anxiolytic and antidepressant-like effect of riparin III in high dosages by intraperitoneal administration (Sousa, Melo et al. 2004). In this work, the anxiolytic-like effect was demonstrated at lower dosage by intravenous administration supported by metabolic profile and behavioral tests. Moreover, It has been speculated that, due its anxiolytic-like effect, riparin III at a 5 mg kg$^{-1}$ dose acts on serotoninergic neurons (Miyata, Shimoi et al. 2007) consisted on a behavior controlled by the amygdala (Fernandez and Gaspar 2012) once previous studies have already indicated the role of amygdala on anxiety, specially its central core”

6. There are too many references in the manuscript. For a short research paper, referencing 118 article is too much. Please keep the most important, relevant and recent reference.

We have retrieved the most ancient references and maintained the most updated.

7. Conclusion section does not read like a conclusion. Some of it can be added to discussion.

Conclusion was rewritten

8. The English should be enhanced in many parts in the manuscript.
The introduction, results and discussion section were rewritten. The modifications are highlighted in green.

9. Page 9 line 208, The Unscrambler not the Unscrubler
Correction has been made

10. Line 215 remove discussion
Correction has been made

11. Figure 2 line 232 AP-o: Anxiogenic Period (closed arms), correct to AP-c
Correction has been made

12. Figures 7 and 8 can be combined
Correction has been made

Reviewer 2: The manuscript describes an important tool to prove the anxiolytic-like effect of riparin III in a more efficient and pragmatic way, using 1H NMR urine metabolic footprint combined with multivariate data analysis. Even though the topic is of interest, the present work needs to be further improved. Especially, the following questions are to be carefully considered.

1 The pharmacological activities of riparin III and its similar compounds (riparin I, riparin II, Riparin A) have been presented in the background section. However, there is no significant effect observed based on the present behavioral tests at the tested dosage of 5mg.kg\(^{-1}\). Is the difference induced by various dosage? or other factors? It's better to give the reasonable explanation.

In this study, the dose administration at 5 mg/Kg has presented a significant difference regarding a few parameters not only by metabolic profile analysis but by Open Field test, as number of groomings and frequency of rearings. The difference regarding research results related to higher dose administration may be due to several facts, such as:

-Route of administration
The intravenous route of administration was used for riparin in this present study, whereas in other studies, as Melo et al., 2006, riparin was administered orally at 25 and 50 mg/Kg. The utilization of different routes of administration may generate different pharmacokinetic parameters as bioavailability, therefore, leading to different results.

-Methodology
The methodology adopted in this study is innovative and unprecedented, once it uses the same animals during different periods (control, anxiogenic and treatment). Therefore, results from different methodologies may generate distinct results.
During this study, riparin was administered once a day for five days. In other published studies, the administration of riparin was either acute (Sousa et al., 2003) or for longer periods (Chaves et al., 2019). Hence, the time of administration has a great influence over results.

2 The comparative analysis between CP and AP groups presented the establishment of model and the significant difference between AP and TP indicated the effect of riparin III. Is it necessary to compare CP and TP when analyzing and discussing the data of behavioral test or the concentration of the metabolites?

This necessity is important in order to verify how similar TP and CP are, once the higher the similarity, the higher the effect of the drug, therefore, establishing a behavior closer to the absence of stress induction. The goal of the metabolic profile study, when comparing TP to CP, is to verify if the TP profile came back to normality (no stress). Figure 5a demonstrate the formation of 3 groups, where TP discrimination is influenced by the riparin III 1H NMR assignment (δ 3.69) which can be observed at the loadings plot, moreover, it is also observed TP samples at the same region of CP, reinforcing the anxiolytic-like effect of riparin III.

3 The positive drugs treated group is not used. It's important for the evaluation of psychopharmacological effects of riparin III.

We understand that a positive control group may be important for a study like this, however, giving the absence of such group, previous studies with riparin that used positive control groups may be used to support that riparin has pharmacological activity

4 1H NMR technique has become a rapid metabolic tool now. What's the reason that only five significant components were differentiated, including cortisol, creatinine, riparin III, 5-hydroxy-L-triptophan and allantoin? How many biomarkers or component type were identified from 3 different periods based on 1H NMR data?

The metabolic profile presented by 1H NMR spectra has detected over 100 metabolites. The multivariate data analysis allows the reduction of these variables (metabolites) into the most influential metabolites or biomarkers responsible for group discrimination. Therefore, it was detected 4 endogenous metabolites and 1 exogenous metabolite (riparin III). The statistical analysis regarding 1H NMR urine metabolic profile, specifically related to the area of each spectrum assignment, has detected on treated animals, an increase of area related to riparin III (δ 3.69) and a decrease related to the cortisol (δ 1.91), indicating an antagonistic effect, or a reduction of cortisol levels due riparin treatment, consequently inhibiting the anxiogenic induction.

5 Fig.5 (d) gave the loadings graphic of urinary metabolites responsible for the differentiation of the 3 analyzed periods (CP, AP and TP). 1H NMR spectrum of urine samples of each period was also necessary to take on the whole metabolite profile of each group.

Figure 5b shows that when there is food restriction, it is more evident the difference between TP (treated with riparin III) and AP (induced stress animals with no riparin III treatment).
This suggests that feeding may reduce stress (Fig. 5c). Other studies has also demonstrated that tryptophan-rich food reduces stress (Haleem 2017, Trujillo, Vieira et al. 2018, Ye, Pitlock et al. 2018), corroborating with our results. Figure 5d shows which 1H NMR assignments has more influence over the groups differentiation

6. Page 11, line 232, AP-o should be AP-c.
Correction has been made

Chaves et al. Reversal effect of Riparin IV in depression and anxiety caused by corticosterone chronic administration in mice. Pharmacology, Biochemistry and Behavior 180: 44–51, 2019


