Author’s response to reviews

Title: Rhaponticum acaule (L) DC essential oil: Chemical composition, in vitro antioxidant and enzyme inhibition properties

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Author’s response to reviews:

Answers to reviewer’s comments

(All modifications included in the text are marked in red)

Dear Editor and reviewers:

Thank you very much for your supervision of the reviewing process of our manuscript entitled "Chemical composition, antioxidant activity and kinetics enzyme inhibition of Rhaponticum acaule (L) DC essential oil" (Reference: BCAM-D-17-00740). We also highly appreciate your carefulness, conscientious, and the broad knowledge on the relevant research fields, since you have given us a number of beneficial suggestions. These comments are all valuable and very helpful for revising and improving our paper. We have studied comments carefully and have made correction which we hope meet with approval. The revised sentences have been marked
Reviewer # 1:

General comments:

1.“The authors evaluated the essential oil composition of this species as it has been previously reported in the literatures (lack of novelty). Moreover, no comprehensive, comparative study was also done on this species.”

Reply: We really admire the reviewer’s broad knowledge.

• According to the literature, there is only work about the chemical composition of the aerial part essential oil of R. acaule published by Boussaada O et al. (Microbiological research 2008). In this work, these authors described the richness of this essential oil in diterpenoids (23.7%), aromatic compounds (23.6%) and oxygenated sesquiterpenes (21.3%), contrary to our essential oil which is characterized by the dominance of sesquiterpene hydrocarbons (74.2%) with germacrene D (49.2%) as major constituent. Indeed, the differences between the chemical compositions of the essential oil obtained from the same species may be attributable to environmental factors (geographical, climatic, and seasonal), development stage and genetic variability. Additionally, chemotypes and individual variability may appear within the same plant species, resulting in the differences in chemical compositions of the raw materials.

• On the other hand, to the best of our knowledge and according to literature, there are no reports on the antioxidant activities of the essential oil from R. acaule. Furthermore, in this work we have investigated for the first time the in vitro α-glucosidase inhibitory activities of R. acaule essential oil as a potential therapeutic target for the treatment and prevention of type 2 diabetes mellitus (DM). In the same way, the inhibitory effect of this essential oil on xanthine oxidase was also conducted in order to search novel natural xanthine oxidase inhibitors which would be beneficial to treat gout and other diseases. Finally and always for the first time, we have showed that the R. acaule essential oil exhibited a high inhibitory effect against pancreatic lipase.
suggesting that it is a promising essential oil for inactivating digestive lipase in order to decrease incidence of common diseases caused by diets rich in carbohydrates and fats.

• Concerning the comparative study on this species, we agree perfectly with this remark. Since the results obtained with the aerial part (Flowers) are very promising, we will consider in the future completing our study on the different organs of this plant in order to establish a consistent comparative study.

2- “The manuscript is lacking the strong discussion of the impact of the work”

Reply: We really appreciate the better suggestion of the reviewer. To better clarify the impact of our work, the discussion was now improved by adding some sentences in the results and discussion section. The new sentences are as follows:

• “Overall, according to the three previous tests, the antioxidant activity found in our results might be attributed to the presence of high percentages of mainly germacrene D (49.2 ± 1.1 %), methyl eugenol (8.3 ± 0.28 %), (E)-β-ionone (6.2 ± 0.18 %) and β-caryophyllene (5.7 ± 0.17 %) (Table 1). In general, Asteraceae species are well documented as natural antioxidants [43] but to our knowledge, no studies were conducted on essential oil of Rhaponticum acaule.”

The new added reference is: “Maisuthisakul P, Suttajit M, Pongsawatmanit R: Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. Food Chemistry 2007, 100: 1409-1418.” Please see the second paragraph of “Phosphomolybdenum assay” part of Results and discussion section of corrected version (line 17 to line 21, page 15) and the new references list.

• “However, further studies are needed to understand the origin of the activity. Particularly, major constituents of the essential oil need to be tested for their antioxidant, anti-α-glucosidase, anti-xanthine oxidase and anti-lipase activities. Furthermore, it is still worthwhile to investigate the other parts of Rhaponticum acaule as a natural source for essential oil composition or phytochemical studies.” Please see the end of the conclusion section of the corrected version (line 14 to line 18, page 20).
Minor comments:

1- “Keywords must be based on alphabet.”

Reply: We thank the reviewer 1 with this remark. As recommended by the reviewer, the keywords were now listed in the revised version in alphabetical order. The new order is as follows: “α-glucosidase; antioxidant activity; chemical composition; pancreatic lipase inhibition; Rhaponticum acaule essential oil; xanthine oxidase”.

2- “Abstract (line 55): typing error for mL”.

Reply: As recommended by the reviewer, the unit (ml) was now corrected and changed by “mL”. Please see line 23, page 2 of abstract section.

3- “The authors should elaborate briefly on the health benefits of main oil to human health by citing appropriate references. This would be interesting for international readers.”

Reply: We really admire the reviewer’s broad knowledge. According to the reviewer’s suggestion, to elaborate the benefits of essential oils on human health, the first paragraph of introduction section was now reorganized and some new sentences were added with the corresponding references. After the modifications, the new paragraph is as follows:

“Essential oil is a hydrophobic liquid extracted from various parts of plants such as flowers, leaves, stems and roots [1]. Due to its aromatic characteristic, essential oil has long been used in the food and cosmetic industries as a flavoring agent [2]. Furthermore, many essential oils exhibit antioxidant properties, which can have a positive effect on biological systems [3, 4] as well as food production by preventing oxidation [5].

For a long time, essential oils have been the basis of traditional medicine in many countries [6, 7]. They are used for many biological properties including bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, and other medicinal properties such as analgesic, sedative, antiinflammatory, spasmolytic, and locally anesthetic remedies [8, 9]. At present, promising researches have been reported using essential oils in medicinal products for human health [10]. These recent work have shown the importance of essential oils to treat other diseases like
respiratory tract, digestive system, gynecological, endocrine, cardiovascular, nervous system, and skin infections. Many of them have shown anticancer activities, too [11].”

The new references added in revised version are as follows:


Please see the two first paragraphs of introduction section (Line 2 to line 15, page 4) of the corrected version and the new references list.

4- “The authors did not give information about software. Please explain completely.”

Reply: We are very sorry for our neglect to detail this section carefully. Several details are now added in “Statistical analysis” section. After modifications, the new paragraph obtained is the following: “The means and standard deviation (SD) of data were calculated from independent experiments. The IC50 (α-glucosidase, xanthine oxidase and pancreatic lipase inhibition) and EC50 (ABTS, reducing power and phosphomolybdenum methods) values were calculated by linear regression analysis and the limits of their confidence intervals were carried out under the normality assumption. Data analysis was carried out using an unpaired Student's t-test. GraphPad Software (USA) was used to fit sigmoid curves models and data were analyzed using a statistical analysis computer software (Graphpad Instat v.3.0a for MacIntosh, San Diego, CA, USA). For all statistical tests, P-values less than 0.05 were considered to be significant.” Please see the new
paragraph of “Statistical analysis” section of the revised version (lines 24-25, page 12 and lines 1-7, page 13).

5- “Source reference for methodology must be provided for Line 17 of plant material section.”

Reply: We agree perfectly with this remark. As recommended by the reviewer 1, to better clarify the essential oil extraction step, some experimental details were now added with the corresponding references. The new paragraph is: “An amount of 100 g of R. acaule aerial parts freshly collected were cut in small pieces and submitted to hydrodistillation for 5-6 h with 500 ml of boiling distilled water using a Clevenger-type apparatus [17] in accordance with the European Pharmacopeia. The distilled essential oil was dried over anhydrous sodium sulfate, transferred to sealed dark vials and stored at 4°C until use. The yield (0.018 %) was calculated based on the fresh weight of the sample.”

The added reference is as follows: “17. Denny EFK. Hydro-distillation of oils from aromatic herbs. Perfum Flavor 1989, 14: 57-63.” Please see the new paragraph (line 11 to line 16, page 6) of “Plant material and essential oil extraction” section and the new references list.

6- “Table 1. Provide standard error.”

Reply: We are very sorry for forgetting to mention the standard error in Table 1. As recommended by the reviewer and according to our analyzes, the standard error values were now added in the revised table 1. Please see new table 1.

7- “Provide mean comparison of Table 2.”

Reply: We agree perfectly with this remark. As recommended by the reviewer 1, all the table 2 was now reorganized. The EC50 values of standard compounds used as positive control were now added and the mean comparison was also performed. The legend of this table was also improved. The corrected version of table 2 is:
Table 2. Antioxidant activity of the R. acaule essential oil.

<table>
<thead>
<tr>
<th>Assay</th>
<th>EC50 values (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RaEO</td>
</tr>
<tr>
<td>+ABTS</td>
<td>0.929±0.118</td>
</tr>
<tr>
<td>++FRAP</td>
<td>0.604±0.021</td>
</tr>
<tr>
<td>+++TAA</td>
<td>0.167±0.019</td>
</tr>
</tbody>
</table>

+ EC50 (mg/mL): effective concentration at which 50% of ABTS radicals are scavenged.

++ EC50 (mg/mL): effective concentration at which the absorbance is 0.5.

+++ EC50 (mg/mL): effective concentration at which the total antioxidant activity (TAA) was 50%.

Effect of Trolox or Ascorbic acid used as standards, were measured in the same conditions than the RaEO. All experiments were performed in triplicate and the results were expressed as the mean ± standard deviation (SD). Statistical comparisons were performed through an unpaired Student's t-test using GraphPad InStat version 3.0a for MacIntosh.

a: p <0.05 vs RaEO

8- “Typing error for mL all text and tables.”

Reply: We thank the reviewer 1 with this remark. As recommended by the reviewer, the unit (ml) was checked in all the text and table 2 and was now corrected by “mL”. Please see all the revised manuscript and corrected table 2.

9- “It is better to provide abbreviation in the foot note of figure 4b.”

Reply: We agree perfectly with this remark. As recommended by the reviewer 1, abbreviations of some words were now added in the foot not of figure 4b. The added abbreviations are as follows: “OOE”: Olive oil emulsion; “NaDC”: Sodium deoxycholate; “TPL”: Turkey pancreatic lipase.
10- “The English usage in certain parts of manuscript is poor and needs to be improved.”

Reply: We thank the reviewer 1 with this remark. As recommended by the reviewer, to improve the English usage in our manuscript, some corrections were now inserted. Please see: (Line 16, page 4); (Line 6, page 6); (Line 16, page 11); (Line 7 and 19, page 12); (Line 24, page 13); (Line 16, page 14); (Line 2, page 15); (Line 8, 21 and 24 page 16) and (Line 7, page 20).

Reviewer # 2:

1- “Title needs to be reframed, as it is not 'inhibition of oil', but it is 'inhibition of some enzyme by oil'.”

Reply: We agree perfectly with this remark. As recommended by the reviewer 2, the title was now reframed and changed by the following: “Rhaponticum acaule (L) DC essential oil: Chemical composition, in vitro antioxidant and enzyme inhibition properties”. Please see the title of the corrected version.

2- “Please re-structure the sentence here any anywhere else in manuscript. Problem is with the term 'inhibition of oil'.”

Reply: We really appreciate the better suggestion of the reviewer 2. To avoid any confusion and to explain that it is the compounds of the essential oil that exert the inhibition and not the inhibition of the oil, the sentence “inhibition of oil” was now re-structured. The corrected sentences are as follows:

• “Enzyme kinetic studies using ............ understand the possible mechanism of inhibition exercised by the components of this essential oil.” Please see the last sentence of “Methods” part of Abstract section (Line 15, page 2).

• “Therefore, the mode of inhibition caused by the essential oil is a mixed-type one, but it seems that it has strong competitive components.” Please see the end of “α-Glucosidase inhibition assay and kinetics study” part of Results and discussion section (Line 2-3, page 17).

3- “Please re-write in a better way.”
Reply: As recommended by the reviewer 2, the sentence “Rhaponticum acaule DC essential oil showed a strong α-glucosidase-inhibitory effect with mixed-type” was now rewritten as follows: “The α-glucosidase inhibition study indicated that the compounds of Rhaponticum acaule DC essential oil are mixed inhibitors” Please see the fourth sentence of “Highlights” section of the corrected version.

4- “Replace 'with' with 'in'. Similar error at all other places also needs correction.”

Reply: As recommended by the reviewer, in the fifth sentence of “Highlights” section, the word “with” was now replaced by “in”.

5- “Please provide common name too.”

Reply: We really appreciate the better suggestion of the reviewer. Accordingly, the plant name cited for the first time in the manuscript is written in full letter as follows: Rhaponticum acaule (L) DC, while for the rest of the report, the name is written as abbreviation: “R. acaule”.

For the same reason, another abbreviation has been standardized throughout the manuscript: Rhaponticum acaule essential oil replaced by the abbreviation: “RaEO”. Please see all the revised version with the new abbreviation list.

6- “Leaves? OR it is a shrub?”

Reply: We agree perfectly with this remark. Rhaponticum acaule (L) DC is one of the most remarkable aromatic plants having an earlier spring flowering, from January to March. It grows wild in rosette on the slopes and in sandy pastures. So, it is not a shrub but it is a vivacious plant, acaule. The aerial part of this plant represents the flowers. Hence, as suggested by the reviewer, the words “aerial parts” were now replaced by the word “flowers” throughout the revised manuscript. Please see line: 6 page 11.

7- “Please provide full details of extraction process. What temp.? Solvent to plant material ratio? Shaking? How much extraction yield?”

Reply: We are really appreciating the reviewer’s comprehensive consideration. As recommended by the reviewer 1 and reviewer 2, to better clarify the essential oil extraction step, some
experimental details were now added with the corresponding references. The new paragraph is: “An amount of 100 g of R. acaule aerial parts freshly collected were cut in small pieces and submitted to hydrodistillation for 5-6 h with 500 ml of boiling distilled water using a Clevenger-type apparatus [17] in accordance with the European Pharmacopeia. The distilled essential oil was dried over anhydrous sodium sulfate, transferred to sealed dark vials and stored at 4°C until use. The yield (0.018 %) was calculated based on the fresh weight of the sample.”

The added reference is as follows: “17. Denny EFK. Hydro-distillation of oils from aromatic herbs. Perfum Flavor 1989, 14: 57-63.” Please see the new paragraph (line 11 to line 16, page 6) of “Plant material and essential oil extraction” section and the new references list.

8- “at what concentration?”

Reply: We thank the reviewer 2 with this remark. As recommended by the reviewer, the Trolox concentration (3.12 - 50 µM) was now added. Please see line 5, page 8.

9- “For this and all other assays, was the oil completely soluble in DMSO? After adding in test medium, was it dissolved fully or partially? Was any surface-active agent used to enhance solubility? Were the 'control' tubes to nullify effect of DMSO (and surface active agent, if used) included in experiment?”

Reply: We really admire the reviewer’s broad knowledge. In our case, before any enzymatic inhibition test, the essential oil was perfectly solubilized in DMSO. This solubility was improved by shaking of essential oil in the ultrasonic bath for a while. This high solubility in this solvent can be explained by the richness of our essential oil in Germacrene D, which is a compound well known by its high solubility in DMSO.

After adding in test medium, our essential oil still remains totally soluble without addition of any surface-active agent. To nullify the effect of DMSO, two control tubes were prepared as following:

Control 1: Pure control having 100% enzyme activity was conducted by replacing the essential oil with DMSO.
Control 2: Blank for pure control having 0% enzyme activity was conducted with DMSO and by replacing the enzyme with buffer.

So, we are very sorry for our neglect to clarify this experimental point carefully. Indeed, in the revised version we have added the corresponding sentences for each enzymatic inhibition test.

Please see:

• Lines 16-20, page 9.
• Lines 4-7, page 11.
• Lines 15-18, page 12.

10- “50' should be in subscript.”

Reply: We thank the reviewer 2 with this remark. As recommended by the reviewer, the number “50” was checked throughout the manuscript and rewritten as subscript. Please see line 20 page 18 and line 4, page 19.

11- “What about the toxicity (to humans/ animals) studies of this oil at the concentrations used in this study? Are any reports available?”

Reply: We really admire the reviewer’s broad knowledge. The essential oil acute toxicity test was performed and the results will be part of another paper that is being prepared.

For this test, laboratory mice (20- 30 g) were used. The animals were subjected to a fasting overnight prior to administration of the essential oil. Different animal groups were treated by oral administration of essential oil at different doses (500, 1000 and 2000 mg/kg). For the first 4 hours after the treatment period, the animals were carefully controlled for any toxic effect. Thereafter, animals were observed for 14 days for any toxic effect or mortality.

Our results showed that toxicity study did not result in any mortality of treated mice and no toxic effects were observed during the study period (14 days). Physical observation indicated that none of them showed signs of toxic effects, such as changes on skin, behavior pattern, salivation, tremors, diarrhea, sleep and coma. Interestingly, no death also was recorded in both the control and treated rats, suggesting that the LD50 for chronic oral dosing with essential oil was much
higher than 2000 mg/kg. According to the obtained LD50 value, it can be suggested that our essential oil should be considered as practically non-toxic in acute ingestion.

12- “In all figures, where are the values of positive and negative controls? How diff. oil conc were decided for diff. enzymes?”

Reply: We really admire the reviewer’s broad knowledge.

• It is recalled once again that all these inhibition experiments were carried out by varying the concentration of essential oil (inhibitor) in order to deduce the type of inhibition with always negative and positive controls. Even if the values of controls are not mentioned in the figures, the IC50 values of the positive controls cited in results and discussion section are sufficient to make the comparison with the samples. The IC50 values of compounds used as positive controls were as follows: Acarbose: 280 ± 10.01 µg/mL; Allopurinol: 2.6 ± 0.16 µg/mL and THL: 0.16 ± 0.001 mg/ml. Please see line 13, page 16; line 13, page 17 and line 21, page 18.

• For the kinetics study of all enzymes studied in this work, the reaction mixture was performed, with increased substrate concentration and in the presence of different concentrations of essential oil. So, the choice of these essential oil concentrations is strictly dependent of the IC50 values obtained with each enzyme (close of IC 50 value). Indeed:

- For the α-glucosidase, the Lineweaver–Burk plots were traced with 7.5, 15 and 30 µg/mL as essential oil concentrations since the obtained IC50 value was 6.7 ± 0.10 µg/mL.

- For the Xanthine oxidase, the Lineweaver–Burk plots were traced with 2.5, 3.75 and 5 µg/mL as essential oil concentrations since the obtained IC50 value was 2.20 ± 0.16 µg/mL.

- For the pancreatic lipase, the residual lipase activity was investigated at different concentrations of essential oil (0.05 to 2.00 mg/mL) since the obtained IC50 value was 0.22 ± 0.002 µg/mL.

13- “Please put values of 'positive control' compounds in the same table.”

Reply: We agree perfectly with this remark. As recommended by the reviewer 1 and the reviewer 2, all the table 2 was now reorganized. The EC50 values of standard compounds used as positive
control were now added and the mean comparison was also performed. The legend of this table was also improved. Please see the new table 2 of the revised version.

Special thanks to you for your good comments.

We appreciate for Editor/Reviewers’ warm work earnestly. We hope that these revisions are satisfactory and the revised version will be acceptable for publication in BMC Complementary and Alternative Medicine.

Once again, thank you very much for your comments and suggestions.

Wish you all the best

Dr. Habib MOSBAH