Author’s response to reviews

Title: Therapeutic Effects of Hydro-Alcoholic Extract of Achillea Wilhelmsii on Indomethacin-Induced Gastric Ulcer in Rats: A Proteomic and Metabolomic Approach

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Author’s response to reviews:

Dear Dr. Karuppusamy Arunachalam

Associate Editor

BMC Complementary and Alternative Medicine

We are very excited to have been given the opportunity to revise and resubmit our manuscript entitled “Therapeutic Effects of Hydro-Alcoholic Extract of Achillea Wilhelmsii on Indomethacin-Induced Gastric Ulcer in Rats: A Proteomic and Metabolomic Approach” (BCAM-D-19-00414R2). We want to extend our appreciation for taking the time and effort to provide such insightful guidance. We carefully considered constructive comments and insightful queries raised by you and reviewers.

We hope that these revisions improve the paper such that you and the reviewers now deem it worthy of publication in the journal of BMC Complementary and Alternative Medicine. Next, we offer detailed responses to your comments.
Before of all, I would to thank reviewers for critically reading and rising valuable points in my manuscript. I am so thankful for spending your time and I am sure this will help me to enrich the paper draft. In the revised version of this manuscript, we used highlighted items in order to highlighting our changes in the manuscript. We have addressed each of your concerns as outlined below.

Reviewers’ comments:

Peter Achunike Akah (Reviewer 3):

Please include all comments for the authors in this box rather than uploading your report as an attachment. Please only upload as attachments annotated versions of manuscripts, graphs, supporting materials or other aspects of your report which cannot be included in a text format.

- Please overwrite this text when adding your comments to the authors. Plant names not properly written. "wilhelmsii" should start with lower case w and not upper case W.

Response: Thank you for this point. We corrected plant names and properly rewrite in revised manuscript which highlighted as yellow.

- In the introduction line 73, the subtype of muscarinic receptor in parietal cells mediating acid secretion is M1 and not M2, hence selective M1 receptor blockers such as pirenzepine are used in ulcer treatment.

The last line (sentence) of introduction is very clumsy and does not reflect the aim/objective of the study.

Response: Thank you for your valuable and insightful comment. For more clarity we have revised this part as follow:

“For this purpose, in the current study, for the first time, we used proteomics and metabolomics-based platforms to study the therapeutic effects and potential treatment mechanisms of Achillea wilhelmsii on indomethacin-induced gastric ulcer in rats”.
“Hence, the aim of this study was to appraise the effects of the hydro-alcoholic extract of Achillea wilhelmsii C. Koch on proteomic and metabolomic profiles of indomethacin-induced gastric ulcer in rats.”.

- 800 mg/kg appears to me as a non-pharmacological dose (very high). What is the LD50 of the extract.

Response: You raised a good point. The acute toxicity of the AW extract was carried out on rats. The rats were fasted overnight before the test, then, the animals were treated orally with AW extract at doses of 200, 400 and 800 mg/kg body weight, respectively and observed for next 24 hours for any sings of toxicity and death. Symptoms for toxicity and death were not observed on each group. Although, we demonstrated in one study that dose of 800 mg/kg of Achillea wilhelmsii significantly reduced acid secretion and ulcer index in comparison to doses of 200 and 400 mg/kg (ref 17). Therefore, we selected dose of 800 mg/kg of Achillea wilhelmsii extract. No death was recorded among the experimental animals within the 24-h observation period after oral administration of up to 800 mg/kg of the crude extracts and their fractions.

- The experimental groupings are should be rearranged for better understanding, eg Gp1 Normal control (Distilled water, dose), Gp 2 Vehicle control (CMC...Dose), Gp 3 Indomethacin 45 mg/kg, Gp 4 Indomethacin (45 mg/kg) + AW (800 mg/kg).

Response: Thank you for your valuable and insightful suggestion. We have changed the experimental grouping to “The rats were allowed one week of acclimatization to their environment, then they were randomly placed into four groups containing 5 rats each, subsequently the animals were grouped as: Group 1: Normal control receiving deionized water (1 ml), Group 2: 1% CMC solution, Group 3: 45 mg/kg body weight of indomethacin (I), Group 4: 45 mg/kg body weight of indomethacin + 800 mg/kg of body weight of AW extract (I+AW)”.
- Why are you specific on indomethacin-induced ulcer? Many ulcers are not indomethacin or NSAIDs-induced. You should also employ other ulcer models eg alcohol, histamine and stress-induced ulcer models for your conclusion be more scientific and acceptable.

Response: Thank you for your valuable suggestion. This study investigated the proteomics and metabolomics effects of the aqueous-ethanolic extract of AW against indomethacin-induced gastric ulcer in rats. Gastric ulceration was induced by single oral of indomethacin (45 mg/kg b.wt.). Therefore, we focused on indomethacin-induced ulcer and discussed the obtained results following ulcer induced with indomethacin. Meanwhile, Specialist investigations, particularly proteomics and metabolomics, are costly, thus, we could not use from the other ulcer model in this study.

Asma Ahmed, Ph.D. (Reviewer 4)

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- Please overwrite this text when adding your comments to the authors

Response: Thank you for your good comment. We have exerted all of your suggestions in revised manuscript.

Sajjad Ahmad, Ph.D (Reviewer 5):

1. There are several English language grammatical and typographical mistakes which should be rectified.

Response: Thank you for this point. we reviewed the manuscript and tried to correct all typographical and grammatical error. English writing was revised by an English native speaker in revised manuscript.
2. Authority name should be provided with the plant name.

Response: Thank you for your valuable comment. We have added the authority name following the plant name in revised manuscript which highlighted as yellow.

3. The name of plant species should be used as per binomial nomenclature rules.

Response: This is a valid and important point. We corrected the name of plant species in accordance with binomial nomenclature rules which highlighted as yellow in the whole revised manuscript.

4. "Single dose of 800 mg/kg were prepared by dissolving the dried extract in distilled water" how was the dissolution carried out as the extract is practically insoluble in distilled water?

Response: You raised a good point. We have extracted my plant using ethanol and water. Extracts were soluble but there was a presence of sedimentation. Particles of the extract were no longer soluble in distilled water. Finally, the extract was dissolved in 5% aqueous dimethyl sulfoxide (DMSO) with shaking and vortexing.

5. Why the flowering branches? Why not other parts?

Response: Thank you for your valuable and insightful comment. We do apologize very sincerely for the mistakes we have made and confusion. We have changed “The flowering branches of the plant” to “The Dried aerial parts of the plant (300 g)” in revised manuscript which highlighted as yellow.

6. How was the plant material dried?

Response: Thank you for this point. We used two different drying methods: 1) Air-drying method and 2) Oven-drying method. The dried aerial parts of the Achillea wilhelmsii (300g) were subjected to air-drying for one week in the laboratory at ambient temperature of 25-30°C and oven-drying at 70°C for 36 h.
7. What was the grade of ethanol, which was used for soaking?

Response: We do apologize sincerely for the mistakes we have made. Based your question, we have changed “The flowering branches of the plant were soaked in a sufficient amount of ethanol at 50°C for 24 hours” to “The Dried aerial parts of the plant (300 g) were soaked in ethanol (50%) for 24 hr and paper filter was used to filter the solute after mixing. The resulting solution was dried using a 40 °C oven for 36 h”. in revised manuscript which highlighted as yellow in (lines 98-101).

8. Why was the 50 oC temperature used for maceration?

Response: Thank you for these points. For more clarity we have explained Maceration method as follow:

300 g of powdered aerial parts of the plant was blended with 50 ml of (ethanol and water) for 24 hours with agitation at room temperature. After, the extracts were taken and filtered by using a 0.45 millipore filter paper. Then, the extracts were concentrated using a rotary evaporator at 40°C under reduced pressure. Finally, the extracts were weighted and stored at -20°C till their usage in the experiments.

9. What was the percent yield of extract?

Response: that’s very important point. The percent yield of extract was 27.45%. We have mentioned this in our previous paper, however, since the main purpose of our current paper is to focus on proteomic and metabolomic effects of this extract, we overlook that in paper.

10. Which protocol was following to count the number of ulcer in the stomach, give reference.

Response: Thank you for your valuable and insightful comment. We do apologize for the mistake. We corrected the protocol of the count the number of ulcer in the stomach in the revised manuscript as following:

“The stomachs were opened along the greater curvature and rinsed with water to remove gastric contents and blood clots and examined to assess the formation of ulcers. The number of ulcers was counted. Mean ulcer score for each animal was expressed as ulcer index (U.I). Ulcer Index= (U/N) ×100, where U is the number of ulcers in the stomachs of each group rats and N is the number of rats in this group. References (18,19) about ulcer index cited in the manuscript which highlighted in text and References.
11. The bibliography should be as per the guidelines of journal

Response: Thank you for valuable point. Based on your advice, we have corrected the bibliography in accordance with the guidelines of journal and amendments performed in the revised manuscript.

12. Each and every reference in the bibliography should contain volume, issue page number etc for instance see reference No. 4.

Response: Thank you for valuable comment. Based on your advice we added the volume, issue page number etc to references in accordance with the guidelines of journal and amendments performed in the revised manuscript.

13. There should be serial dilutions of the extract to be screened rather than a single concentration

Response: You raised a good point. We demonstrated in one study that dose of 800 mg/kg of Achillea wilhelmsii is the better than doses of 200 and 400 mg/kg for the therapy of indomethacin – induced gastric lesions. In this study, we assessed the possible anti-ulcerative activity of Achillea wilhelmsii extract by measuring the protective index, mucus secretion, NP-SH (non-protein sulphydryl compounds) and lipid peroxidation in gastric tissue in addition to examination macroscopic features and histopathological markers. In this study, Achillea wilhelmsii decreased the extend of ulcers compared to control group. Moreover, the results were revealed that Achillea wilhelmsii extract significantly reduced acid secretion in doses of 400 and 800 mg/kg compared with indomethacin control group. A statistically significant difference in acid secretion and ulcer index was observed by Achillea wilhelmsii extract 800 mg/kg in comparison to control group. Meanwhile, we have added sentence” We selected dose of 800 mg/kg of Achillea wilhelmsii extract based on the findings of our previous studies on Achillea wilhelmsii” and its reference to Plant extract: hydro-alcoholic extraction in method section (lines 88-89) in revised manuscript and highlighted as yellow. Finally, we selected the dose of 800 mg/kg of Achillea wilhelmsii for present study.
14. The dendrogram provided is not clear

Response: Thank you for valuable and insightful comment. Since, not only our aim of this study was to appraise of the effect of the hydro-alcoholic extract of Achillea wilhelmsii C. Koch on proteomic and metabolomic profiles in indomethacin-induced gastric ulcer in rats, but also there was several published studies that evaluated the chemotaxonomic affinity and relationship amongst 20 ecotypes of A. wilhelmsii, using hierarchical cluster analysis. hence, we have no perform dendrogram analysis for Achillea wilhelmsii in current study. We only write sentence “Achillea is a genus belonging to the Asteraceae family which embraces more than 100 species worldwide” in the manuscript.

A.H.M. Khurshid Alam, PhD (Reviewer 6):

Mehdi et al described the effects of Hydro-Alcoholic Extract of Achillea Wilhelmssii on Indomethacin-Induced Gastric Ulcer in Rats. Their findings were interesting. However, the authors need to address the following queries before accept it into this journal.

1. The author mentioned Hydro-alcoholic extract of Achillea Wilhelmsii. I do not think it was Hydro-alcoholic extract, because the flowering branches of the plant were soaked in a sufficient amount of ethanol at 50°C for 24 hours and during this period, it was shaken alternatively (page 8, lines 97-99). Then the ethanolic extract was just dissolved with water to administered........ I think it does not mean the extract was Hydro-Alcoholic. Author must clarify this.

Response: Thank you for your valuable point. We do apologize sincerely for the mistake. For more clarity we have change this sentence “The flowering branches of the plant were soaked in ethanol at 50°C for 24 hours and during this period, it was shaken alternatively. The solution obtained was then filtered (with Whatman No. 1 filter paper) and was placed in water bath (40°C) for 36 hours”

To

“The Dried aerial parts of the plant (300 g) were soaked in ethanol (50%) for 24 h and paper filter was used to filter the solute after mixing. The resulting solution was dried using a 40 °C oven for 36 hr”. The amendments performed in the revised manuscript in (lines 98-100).
2. The authors checked mRNA expression of some genes but not protein by real time PCR. It should be corrected throughout the manuscript.

Response: You raised a good point. We have changed “proteins” to “genes” in throughout revised manuscript which highlighted as yellow.

3. I do not find any control (positive) drug, which is important to compare the effect of the extract of Achillea Wilhelmsii.

Response: Thank you for your attention to this point. We performed two pilot studies that in these studies we used ranitidine as standard drug to prove the efficacy of the extract. Ranitidine is an H2 histamine receptor antagonist that works by blocking histamine and thus decreasing the amount of acid released by cells of the stomach. In Our previous study, the rats wistar were divided into elven groups (each 8), rats in group 1 received indomethacin with single dose of indomethacin as gavage, rats in group 2 received carboxymethyl cellulose 1% as drug control and rats in group 3 received indomethacin single dose 45 mg/kg and ranitidine with dose 150 mg/kg as a standard drug to prove the efficacy of the extract. Therefore, we did this in previous pilot studies and approved the efficacy of the extract. The findings from the pilot studies showed that both the Achillea wilhelmsii extract and ranitidine (150 mg/kg body weight) for three days after single dose of indomethacin increased the therapeutic indexes relative to group receiving indomethacin alone. Meanwhile, the human dosage of ranitidine used in market as medicine is 150 mg/kg body weight. Thus, we have used from our previous findings for designing current study.

4. Did the author check sub-acute toxicity test of the extract to select the dose?

Response: You raised a good point. The acute toxicity of the AW extract was carried out on rats. The rats were fasted overnight before the test, then, the animals were treated orally with AW extract at doses of 200, 400 and 800 mg/kg body weight, respectively and observed for next 24 hours for any sings of toxicity and death. Symptoms for toxicity and death were not observed on each group. No death was recorded among the experimental animals within the 24-h observation period after oral administration of up to 800 mg/kg of the crude extracts and their fractions. Although, we demonstrated in one study that dose of 800 mg/kg of Achillea wilhelmsii significantly reduced acid secretion and ulcer index in comparison to doses of 200 and 400 mg/kg (ref 17). Therefore, we selected dose of 800 mg/kg of Achillea wilhelmsii extract.
Again, we appreciate the opportunity to revise our manuscript for consideration in your journal. We hope our revision meet with your approval.

Fatemeh Goshadrou,

Best Regards