Author's response to reviews

Title: Antinociceptive principle from Curcuma aeruginosa

Authors:

Chowdhury F Hossain (faiz@ewubd.edu)
Mohammad Al-Amin (2006alamin@gmail.com)
Abu Sadat M Sayem (sayem062@yahoo.com)
Ismail H Siragee (ismailsiragee@gmail.com)
Asif M Tunan (tunan SwiftUI@yahoo.com)
Fahima Hassan (trishi_8a@hotmail.com)
Md Mohiuddin Kabirb (mmkabir@ewubd.edu)
Gazi Nurun N Sultana (nngazi@gmail.com)

Version: 4 Date: 15 May 2015

Author's response to reviews: see over
Dear Editor:

Thank you for sending our manuscript for corrections based on the reviewer’s comments. We made the necessary correction as recommended by the two reviewers. All queries of reviewers are answered and given below. Now I am uploading the corrected version of the manuscript herewith.

I would appreciate very much if you kindly consider it for publication.

Thank you.
Sincerely,

Chowdhury Faiz Hossain, Ph.D.
Chairperson and Professor,
Department of Pharmacy
East West University
A/2, Jahurul Islam City, Aftabnagar, Dhaka-1212,
Bangladesh.

Phone: 09666775577 ext. 211 and 212
Reviewer's report

Title: Analgesic principle from *Curcuma aeruginosa*.

Version: 3 Date: 8 March 2015
Reviewer: Patricia Fernandes

Reviewer's report:
Major Compulsory Revisions:

- there are some typographical errors that must be corrected.

Response: Manuscript has been scrutinized for typographical errors and corrected.

- which is the amount of pure substance found in the extracts? this should be the dose used for pure substance and not a dose of 20 and 40 mg/kg. it means that germacrone is 10% of the crude extract?? At this high dose, it is expected to observe some effect.

- Authors must show, and discuss, the results obtained with doses equivalent to the amount of germacrone found in extracts. Without this data it is improper to indicate that the effect of the extract is due to this substance.

Response to the above two comments:

Pure active metabolite, germacrone (1), of 2.0 gm was purified from 175.0 gm of crude extract which represents a yield of 1.14%. The extract obviously contains other germacrone derivatives which might contributed the activity of the crude extract and its subtractions. Adjacent fractions of SF. 3 and the mother liquid of germacrone crystals also contained some amount of germacrone. We did not determined the content of germacrone in *C. aeruginosa* which need an analytical work. The purpose of the present research was separation and identification of the major active metabolite of *C. aeruginosa*. So it is not relevant to determine the dose based on the content of germacrone in *C. aeruginosa*. Doses were selected on the basis of reported toxicity profile and dose of positive control. The result also demonstrated dose dependency in the activity which are statistically significant.

- tables should be changed by graphs. It is better to observe the effect than in table.

Response: All tables have been changed by the graphs as recommended.

- formalin-induced licking and contortions models are not models of analgesia. Both are models of ANTINOCICEPTION. In animals we observe antinociceptive activity. This must be changed all over the Ms.

Response: Manuscript has been corrected as recommended.
Minor Essential Revisions

- why authors used two different anti-inflammatory drugs as controls in contortion and formalin-induced licking response?

Response: Use of aspirin in antinociception assay gives two of response early phase (1\textsuperscript{st} phase) and late phase (2\textsuperscript{nd} phase) which can be interpreted to two different kind of mode of actions. That is why we used aspirin in formalin-induced licking methods.

On the other hand, for acetic acid induced writhing method, diclofenac is widely used as positive control.

- the references used to contortions and formalin-induced licking are not the original ones. Authors should add the original ones. Theses references were the first to padronize the method. after them several other papers did modifications. and these modifications can be cited.

Response: The references for acetic acid induced writhing and formalin induced licking method have been changed and more authentic and original references have been included.

- doses of 200 and 400 mg/kg are too high and do not correlate with the popular use. two doses are not enough to demonstrate a dose response curve. Why authors did not show another dose (i.e., 100 or 500 mg/kg)?

Response: We selected the doses in accordance with the toxicity profile and the positive control. As the doses of 200 and 400 mg/kg were more comparable with the positive control and did not exert any toxic effect, therefore we selected these doses. If we use 100 mg/kg dose, then the result would not be significance and if we select the dose of 500 mg/kg, then it will produce more activity. Therefore we selected two doses that are more effective and comparable with the positive control and have no toxic effect.

-the effect of extract in contortion model was low. only 37.5% inhibition to 200 mg/kg. this dose is 20 fold higher than the positive control group.

Response: As the extract contains numerous inactive metabolites together with limited number of active compounds and their derivatives which exerts activity. Therefore it is necessary to choose higher doses for the extract that would be comparable with the positive control and do not exert any toxic effect.

In addition we decreased the amount gradually after fractionation and selected 10, 20 and 40 mg/kg doses for the active constituents. Furthermore we used 10 mg/kg dose for the diclofenac and 100 mg/kg dose for the aspirin.
Reviewer 2:

This study uses a plant extract from Curcuma aeruginosa, its fractions and an isolated compound to reduce the nociceptive response after acid acetic or formalin injection. This study is interesting in the field of nociception. However, there are several points need to be concerned.

- Major Compulsory Revisions

1. In the animal section of method, it is described that it was used both sex in the experiments of nociception. It is known that the inclusion of females is mandatory in clinical but not in preclinical studies. In fact, some studies have demonstrated the differences in the feeling of nociception and pain in animals and in humans depending on the sex or gender, respectively, beyond the knowledge that nociception is mediated through different mediators in male and female mice and probable involves sex hormones. Moreover, there are sex differences in many neurotransmitter systems.

   **Response:** The reviews observation is reasonable. But in general screening purpose, it has been used of both sex animals that is evident in many peer reviewed published articles, e.g. in the reference # 16.

2. It is necessary to review the methodology of tests. The method used to quantify the writhing is not the recommended one, which is a complete abdominal muscle contractions and hind paw extensions, besides the time of observation needs to be for 30 minutes. The authors observed for only 10 minutes the number of writhing, and the full writhing was counted as two half writhings taken as one full writhing. Also, the positive control group was diclofenac, which is not the most used drug for this purpose.

   **Response:** More references have been cited for the writhing method. The time and positive control are recommended by these references (Ref # 15, 16, 17 and 18).

3. In the formalin-induced hind paw licking sections of method, why did the authors not proceed the experiments with SF. 1 to SF. 5? The authors may do it to complete the results data.
Response: We used acetic acid induced writhing method for screening the antinocicetive principle and therefore all crude extracts, theirs fractions, sub fractions and isolated pure compound are tested, while the formalin method was used to distinguish whether the pure compound, germacrone (1), acts centrally or peripherally. Therefore it was not essential to check licking of SF. 1 to SF. 5.

4. Also in the method section, why did the authors proceed the experiments with 200 mg/kg of VLC fractions in the acetic acid induced writhing and in the formalin-induced hind paw licking they used 100 mg/kg of VLC fractions?

Response: We had data for 100 mg/kg of VLC fractions in the acetic acid induced writhing method. So we replaced 200 mg/kg by 100 mg/kg in the manuscript as suggested.

5. The authors need to review the discussion about the formalin test. Studies have shown that non-steroidal anti-inflammatory drug has no effect on the first phase of the formalin test, even though the acetylsalicylic acid (ASA) and paracetamol were antinociceptive in both phases. It is contrary to the results obtained by this study, which reported a significant effect of aspirin just in the early phase of the formalin test.

Response: The results and discussion section has been rewritten as suggested.

6. Furthermore, the authors need the rewrite the first sentence of the second paragraph. The discussion is not correct since in the early phase of the formalin induced licking is due to a direct effect on nociceptors, but it is not a test to conclude the involvement of the central nervous system, and it is neither correct to affirm that the late phase only indicates that a drug is a NSAIDs because studies has shown that not only NSAIDs have effect in this phase, but also analgesic opioids.

Response: The results and discussion section has been rewritten as suggested.

7. In a previous study (reference 4), it was observed that only the chloroform extract of C. aeruginosa suppressed the licking activity of the late phase in the formalin test in mice, but not the methanol extract of C. aeruginosa. In the present study, however, the authors show that the methanol extract exhibited inhibition of licking in both the early and the late phase. How can these differences can be justified?

Response: In this reference literature, the authors extracted first the plant materials with a nonpolar solvent chloroform, then further extraction was conducted by methanol and water successfully. Therefore, their low polar antinociceptive compound was extracted in the chloroform, but we use methanol for our first extraction which has a more cell penetrating and extracting power and commonly extract both polar and nonpolar secondary metabolites from the
plant. So we fractionated that methanol extract and we discovered the active principle in the nonpolar hexane fraction. In this ways it can be justified.

8. The authors need to review the conclusion since it is not suitable to affirm that germacrone acts on the central nervous system.

Response: The conclusion section has been corrected as suggested.

9. The molecular mechanism of antinociception action of methanol extract from Curcuma aeruginosa, its fractions or the isolated compound germacrone is still unclear. This should be clarified by additional examination.

Response: It is clarified by additional discussion as suggested.

- Minor Essential Revisions

1. Title needs to be re-written. Since this work is yielded in experimental protocols, it is necessary to change the term "Analgesic principle" for "Antinociceptive principle". The changes are also necessary throughout the text.

Response: Manuscript has been changed as per recommendation.

2. In the section methods of the abstract, the authors used the expression "20, 40 mg/kg of 1", but the symbol 1 has not been described yet and it is not clear what it corresponds to. It is necessary to specify the term "of 1" before the authors cite it.

Response: Abstract has been corrected as suggested.

3. In the section methods of the abstract, the authors should include which groups were used for negative and positive control groups. It is also necessary to include the doses used for Fr 1-5 and Sf 1-5 in the nociceptive tests.

Response: Abstract has been corrected as suggested.

4. In the section methods and results of the abstract, the authors used the expression "compound 1". Sometimes the authors use the term "of 1", in other times the authors use the term "compound 1", and in other times the authors use the term "germacrone". It is necessary to standardize the terms.

Response: Corrected has been made throughout the text as recommended.
5. In the section results of the abstract, the authors affirm that "Fr. 1 was found to have the most potent analgesic activity", but the data was not showed in results comparing the 5 fractions. The same was observed with Sf. 1-5. It is necessary include data.

**Response:** Abstract has been corrected as per the recommendation.

6. In the section results of the abstract, the unique data showed was related to the methanol extract of Curcuma aeruginosa and to the germacrone, but the authors did not do the correspondence between the percentages and the doses used for both the acid acetic and the formalin test. It is necessary to complete the results data.

**Response:** Correlation between percentages and the doses used for both the acid acetic and the formalin test has been discussed and data has been included.

7. In the section conclusion of the abstract, the authors affirm that "Germacrone showed a potent analgesic activity that acts on the central nervous system", but in the methods section, the authors contradict this sentence saying that the tests chosen were used "to investigate a peripherally acting analgesic drug". In fact, neither the acid acetic nor the formalin test are methods to identify the involvement of the central nervous systems. To affirm that, the authors need to develop others experiments like hot plate or to administer substances via intra-thecal to test the supra-spinal mechanisms. It is suggested to change the sentences or to proceed the experiments.

**Response:** According to the literatures, the acetic acid induced writhing test is most commonly used for the peripheral antinociceptive test. The inhibition of both phased is a indicative for drugs that have central activity while the inhibition of the second phase is mediated by peripheral antinociceptive and also suggest the compounds might have anti-inflammatory activity. We discussed and corrected the manuscript according to the literature and cited all these literature in the reference section (ref # 18, 22 and 24).

8. In the key words of the abstract, the word "Zingiberaceae" was not cited in the text. The authors should insert the term in the abstact to justify its use as a key word. The same was observed for "Cyclic sesquiterpene".

**Response:** Abstract has been corrected as suggested.

9. In the background section, the authors comment the activities demonstrated for Curcuma aeruginosa in the literature, but there are activities that were put out of the text. It should be deserved attention for the papers bellow:

Functional food quality of Curcuma caesia, Curcuma zedoaria and Curcuma aeruginosa endemic to Northeastern India. Liu Y, Roy SS, Nebie RH, Zhang Y,


Response: we included three of the suggested literatures in the manuscript.

10. In the background section, the authors commented that germacrone, furanodiene, curcumenol, zedoarol, zedoarondiol, zedoalactone A, zedoalactone B, isocurcumenol, and isofuranodiene are the major chemical constituents isolated from the rhizomes of C.aeruginosa. Why the authors did not proceeded experiments with these compounds to certify that germacrone is in fact the principle antinociceptive compound?

Response: Objective of our research was to discover the bio-active principle of Medicinal plants of Bangladesh. That is why, we conducted a bio-assay guided separation and identified the most active analgesic compound of C. aeruginosa. Therefore, we did not try to get those natural products and test their activities.

11. From the method to results and discussion sections, the authors should standardize the presentation form of the symbols, putting or not space between the symbols and the numbers.

Response: Manuscript has been corrected and scrutinized throughout the manuscript.

12. In the acetic acid induced writhing and formalin-induced hind paw licking sections of method, it is necessary to change the word "and" between the doses for "or" between them.

Response: Methods has been corrected as suggested.
13. In the results and discussion section, the references 10 and 14 are not the best ones to discuss that "Acetic acid induced writhing is a useful method for investigating peripherally acting analgesic drug leads". Similarly it was observed with the references 11, 12 and 13 to discuss about the formalin test. It is necessary to change for a more appropriated ones.

Response: The references 10 and 14 has been eliminated and more authentic references have been included and manuscript has been corrected more perfectly in accordance with the published literatures.

14. In the results and discussion section, the tables 1 and 2 need to be cited early in the text.

Response: We changed the table 1 and table 2 and showed the data as bar diagram according to the 1st reviewer and included them where it would be more suitable.

15. It is already known that germacrone also exerted anti-inflammatory activity in carrageenin-induced hind paw edema in rats (Claeson et al., 1993). This information could be inserted in the discussion section.

Response: We include the recommended literature (ref # 13) and discussed in accordance with the finding of that manuscript.

16. There are not legends for the figures 1 and 2.

Response: Legends were included as recommended.

17. The format of the tables needs to be reviewed, they are out of the format recommended by the instructions for authors.

Response: The tables has been changed by graphs in response to the recommendation of the second reviewer and format has been corrected.

18. The references need to be also reviewed, they are out of the format recommended by the instructions for authors.

Response: The reference section has been corrected according to the guideline of the manuscript and scrutinized.

19. It is recommend to edit the manuscript by a native English speaker.

Response: The manuscript has been scrutinized by a native English speaker Dr. Edward L. Organ, Former Chair, Genetic Engineering and Biotechnology, East West University, Dhaka, Bangladesh.
Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Not suitable for publication unless extensively edited

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests